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STUDIES ON THE MECHANISM OF ACTION
OF DRUGS ON SMOOTH MUSCLE

by



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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

FALL, 1978

DEDICATED TO

THE SPIRIT OF W. HEATH ROBINSON

ABSTRACT

The effects of diazoxide on airway smooth muscle were investigated in vitro and in vivo. Using guinea pig tracheal chain preparations, diazoxide was shown to reduce the inherent tone of airway smooth muscle and to antagonize histamine-induced increases in tone. The actions and potency of diazoxide were similar to those of aminophylline. Both diazoxide and aminophylline were observed to potentiate the responses to isoproterenol (INA) or salbutamol. The actions of diazoxide and aminophylline were shown to be unaffected by the presence of propranolol in concentrations sufficient to produce β -adrenergic blockade. Diazoxide, unlike either aminophylline or INA, was observed to produce a slight contraction before inducing relaxation.

In vivo, the effects of diazoxide on the total resistance of the lung to inflation (R_{TL}) were investigated. In addition, the specific effects of diazoxide on lung compliance (C_L) and flow resistance (R_L) were also determined. When R_{TL} was measured, diazoxide was shown to be an effective bronchodilating agent capable of inhibiting histamine-induced increases in R_{TL} . However, the bronchodilating effects of diazoxide were preceded by an initial increase in R_{TL} . The bronchodilation caused by diazoxide was shown to involve actions in both the large and small airways.

The initial increase in R_{TL} produced by diazoxide was shown to involve a marked increase in R_L and a slight to moderate decrease in C_L . The increase in R_L could be selectively blocked by pretreatment with mepyramine, suggesting histamine-like action or histamine release in

the large airways. The decrease in C_L was selectively abolished by pretreatment with indomethacin or ASA, suggesting a prostaglandin-like action or release in the small airways. Neither vagotomy nor pretreatment with atropine affected the initial responses to diazoxide. Thus, the increase in R_L and the decrease in C_L are unlikely to involve vagal reflex activity or the release of acetylcholine.

It was concluded that diazoxide effectively relaxes airway smooth muscle, but that its relaxant properties are preceded by an initial constriction of airway smooth muscle.

ABSTRACT

The effects of hexamethonium, DMPP, nicotine, trimethaphan and mecamlamine on the binding of agonists to the muscarinic receptor of acetylcholine were investigated. The dissociation constant (K_D) was used as an index of binding, and was determined for the agonists, acetylcholine, acetyl- β -methylcholine and carbachol. The values of K_D were calculated according to methods described by Furchgott (1966) and Parker & Waud (1971), using phenoxybenzamine (POB) as the irreversible antagonist. The percentage of receptors blocked by POB and tissue sensitivity to an agonist, before and after POB-blockade, were also calculated.

Hexamethonium was observed to produce a statistically significant increase in the K_D of carbachol, but did not affect the K_D 's of the other agonists. The effect of DMPP on the K_D 's of agonists could not be calculated by the methods used in these investigations because of an increase in the apparent R_{max} after POB-blockade when DMPP was present in the bath. The other ganglionic agents produced no significant changes in the K_D of the other agonists tested.

The effect of hexamethonium on the K_D of carbachol was explained in terms of a model in which the receptor can exist in more than one conformation and in which each conformation had different binding affinities for an agonist. As hexamethonium did not appear to alter tissue sensitivity or to change the percent of receptors blocked by POB, it was suggested that more receptors were caused to exist in a high affinity conformation due to an indirect effect at the receptor site. It was also

suggested that the selectivity of this effect might be related to the preferred conformations of the agonists, acetylcholine, acetyl- β -methylcholine and carbachol, in solution.

FOREWORD

This thesis is concerned with studies of pharmacological effects on smooth muscle and can be conveniently divided into two parts. The effects of diazoxide on airway smooth muscle are presented in Part I. Part II is concerned with a study of the binding of drugs to the muscarinic receptor for acetylcholine using longitudinal muscle strips of guinea pig ileum.

ACKNOWLEDGEMENTS

I wish to thank Dr. D.F. Biggs for his suggestions and help, and also for his thoughtfulness and patience, during the course of these investigations.

I would also like to thank Mr. C. Ediss for his help with the electronics and computer programming used in these investigations.

Finally, I would like to acknowledge the financial assistance received from the Medical Research Council of Canada during the course of this work.

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PART I

THE EFFECTS OF DIAZOXIDE ON AIRWAY SMOOTH MUSCLE

INTRODUCTION

This section of this thesis is concerned with the determination of the effects of diazoxide on the respiratory system and the identification of the sites and mechanisms involved in producing these effects. In order to place these investigations into perspective, this introduction will briefly review the following topics:

1. The anatomy and normal physiology of airway smooth muscle, including the reflexes affecting smooth muscle tone (A);
2. The actions of agonists (both constricting and dilating) and antagonists on airway smooth muscle (B & C);
3. The basic theory of lung mechanics which makes possible the independent measurements of flow resistance and dynamic lung compliance (D); and
4. Some of the methods which have proven useful in the study of airway smooth muscle and respiratory function (E).

A. ANATOMY AND PHYSIOLOGY

The purpose of the respiratory system is to provide oxygen to the body and, at the same time, to remove carbon dioxide. In order to achieve these purposes, two separate processes are involved in respiratory function (Guyton, 1971). The first process is pulmonary ventilation which can be defined as the movement of air into and out of the lungs. The second process is diffusion of oxygen and carbon dioxide between the alveoli and pulmonary circulation. In this study, only effects on pulmonary ventilation were examined and therefore the second process will not be considered further.

The flow of air into and out of the lungs is essentially determined by the actions of the muscles used in breathing, the tone of the smooth muscle within the airways, and total pulmonary compliance. Alterations of smooth muscle tone in various parts of the airways may produce different effects on pulmonary ventilation (Mead & Whittenberger, 1953; Mead, 1961; Nadel, 1965; Collier, 1968), and these can be used to divide the airways into two relatively distinct groups.

The first group is the large airways including the trachea and larger bronchi (see Figure 1). The second group consists of the small airways which extend from the terminal or respiratory bronchioles to the alveoli. Most of the resistance to air flow occurs in the larger airways, whereas the major volume of air is contained in the small airways (Brody, DuBois, Nisell & Engelberg, 1956; Nadel, 1965; Collier, 1968; Colebatch, 1970).

As most of the air is contained in the small airways, it is not surprising that changes in muscle tone of these airways affect the volume of air moved through the respiratory system. As these airways are occluded, alveoli may collapse and more force will be required to expand the lungs (Mead, 1961). That is, a smaller change in volume per unit of pressure change will be effected. Lung compliance (C_L), defined as the change in volume per unit of pressure change, can therefore be used as an index of small airway constriction. It should be noted that lung compliance measured during respiratory cycles is more correctly referred to as dynamic lung compliance. However, as static lung compliance was not measured in this study only the term 'lung compliance' will be used for the sake of convenience. Constriction of the small airways produces a fall in compliance whereas dilation of the airways results in an increase

in C_L (Mead & Whittenberger, 1953; Mead, 1961; Karczewski & Widdicombe, 1969b; Colebatch, 1970; Drazen & Austen, 1974). Also, measurements of C_L in vivo are affected by factors such as the compliance of the chest wall and therefore reflect total pulmonary compliance.

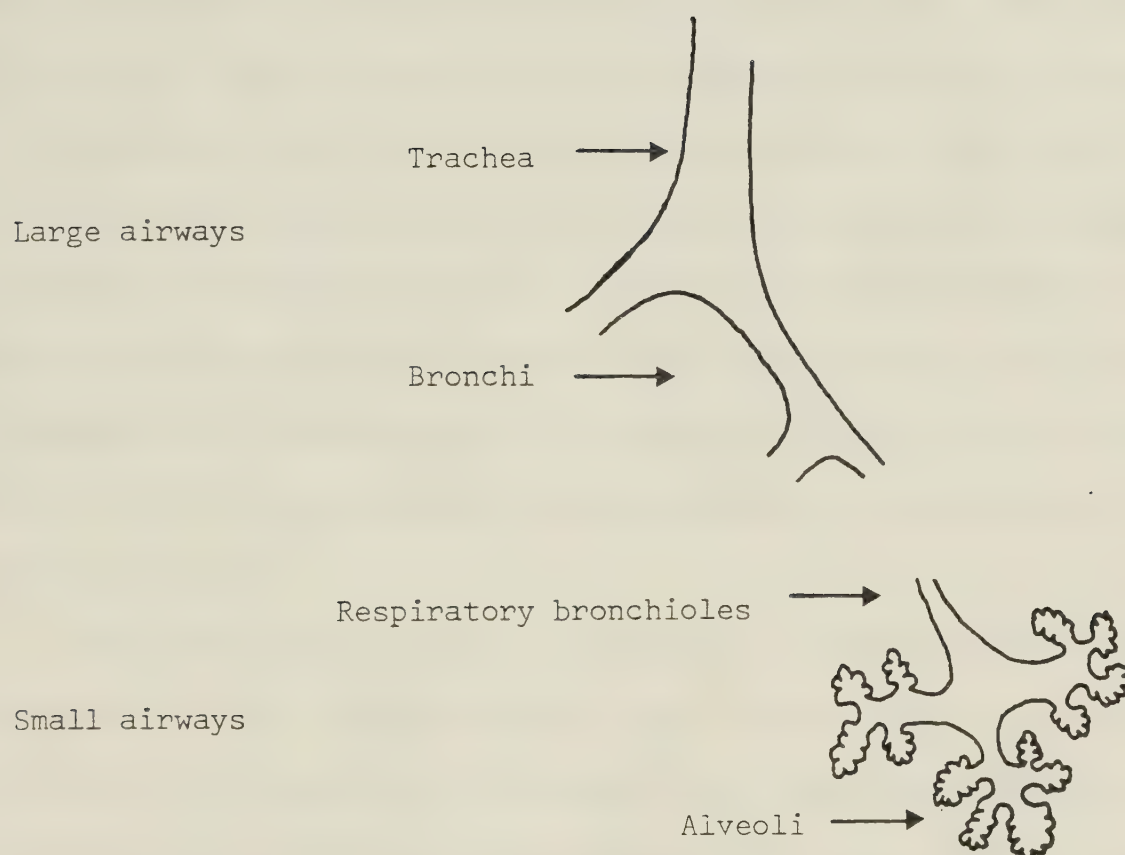


FIGURE 1. The large and small airways.

In contrast, changes in the calibre of large airways greatly affect the rate of airflow but have little overall effect on the volume of air flowing within the system. These changes involve non-elastic or resistive forces and are referred to as changes in flow resistance, R_L , or conductance, $1/R_L$ (Mead & Whittenberger, 1953; Mead, 1961; Colebatch, 1970; Drazen & Austen, 1974).

Actions on airways of intermediate size affect both C_L and R_L in varying degrees related to their size (Nadel, 1965).

The airways are supplied with blood from the bronchial circulation and the pulmonary circulation according to their size. Thus large airways are supplied by the bronchial circulation and small airways by the pulmonary circulation. Therefore, injections of drugs into the bronchial arteries primarily affect the large airways and R_L , whereas pulmonary administration tends to localize the initial effects in the small airways and alter C_L (Nadel, 1965; DeKock, Nadel, Zwi, Colebatch & Olsen, 1966). Dautrebande (1970) concluded that $\geq 99\%$ of fluid injected into the pulmonary artery was directed to the alveoli rather than the bronchi.

The airways are densely innervated by both sympathetic and parasympathetic fibers (Dixon & Brodie, 1903; Miller, 1937; Widdicombe, 1963). In addition to sympathetic nervous activity, sympathetic tone is also influenced by circulating catecholamines (Widdicombe, 1963; Simonsson, Svedmyr, Skoogh, Andersson & Bergh, 1972). It is well established that the predominant sympathetic effect in the airways is bronchodilation which is mediated by β_2 -adrenergic receptors (Widdicombe, 1963; Simonsson et al., 1972; Douglas, Dennis, Ridgway & Bouhuys, 1978).

Blockade of these receptors can produce bronchoconstriction, especially in asthmatic patients (see review by McDevitt, 1978).

The existence of α -receptors in airway smooth muscle has been a controversial subject. The arguments for their existence have largely centered on four types of observations:

1. After β -blockade, α -agonists have been observed to contract isolated airway smooth muscle preparations (Fleisch, Maling & Brodie, 1970; Simonsson et al., 1972).
2. After β -blockade, α -agonists have been observed to produce bronchoconstriction in vivo in man (Bianco, Griffin, Kamburoff & Prime, 1974).
3. α -Antagonists have been observed to potentiate the relaxation induced by β -agonists in isolated tissues (Nousiainen, Arnala, Airaksinen & Kokkola, 1977), and
4. Clinically, α -antagonists have been observed to potentiate the bronchodilation induced by β -agonists in man (Bianco et al., 1974; Palmer, Gaddie & Skinner, 1974; Patel & Kerr, 1975). Patel & Kerr (1975) also reported that a combination of thymoxamine (α -blocker) and salbutamol (β_2 -agonist) was effective in three patients who did not respond to salbutamol alone.

Not all workers have been able to confirm such observations. Foster (1966) could detect no contraction of isolated guinea pig tracheal preparations when noradrenaline was tested during β -blockade. It is possible that the weak local anesthetic effects of propranolol could interfere with detection of such contractions as they have been reported to be, at most, only 10-20% of the maximum that can be achieved with carbachol

(Simonsson et al., 1972). Guirgis & McNeill (1969) using isolated human bronchial tissues and Stone (1973) studying human subjects in vivo also failed to detect any α -adrenergic activity. Similar results were obtained by Cabezas, Graf & Nadel (1971), when investigating the sympathetic and parasympathetic control of the airways in dogs.

Several alternative explanations for the observations listed above have been proposed. It has been suggested that the increases in tone after β -blockade could be the result of unimpeded cholinergic activity (Guirgis & McNeill, 1969; Cabezas, Graf & Nadel, 1971; Simonsson et al., 1972). The ability of α -blocking agents to potentiate the dilation induced by β agonists may be explained by one or more of the following factors:

1. Antihistaminic effects of α -blockers (Guirgis & McNeill, 1969);
2. Inhibition of catecholamine uptake mechanisms by α -blockers (Foster, 1966; Guirgis & McNeill, 1969);
3. Direct relaxant effects of α -blockers, e.g. phenoxybenzamine (Guirgis & McNeill, 1969);
4. Increased catecholamine release by α -blockers (Guirgis & McNeill, 1969).

Interpretation of the data from these investigations is further complicated by the possibility of species differences, differences between large and small airways in the same species, and also by the suggestion that α -receptor development or function might be age-dependent (Fleisch, Maling & Brodie, 1970). At present, the data concerning the existence of α -receptors remains inconclusive. However, the more recent literature does suggest that α -receptors are present in the airways in man.

Parasympathetic fibers are involved in both bronchoconstriction and bronchodilation. Efferent fibers form a complex neural network around the trachea and large bronchi, but do not appear to extend to the smaller airways (Dixon & Brodie, 1903; Miller, 1937). Stimulation of most efferent fibers results in constriction of the large airways (Cabezas, Graf & Nadel, 1971). Afferent vagal fibers originate in the alveoli and are believed to be indirectly involved with bronchodilation (Miller, 1937; Nadel, 1965; Karczewski & Widdicombe, 1969a). Generally, however, vagal constrictor pathways exert the predominant influence on the airways (Nadel, 1965; Cabezas, Graf & Nadel, 1971).

Drugs and other agents can affect airway smooth muscle directly, reflexly, or through a combination of direct and reflex actions. Direct actions will be reviewed first.

B. DRUGS ACTING DIRECTLY

Endogenous or exogenous acetylcholine is known to produce marked constriction of airway smooth muscle (McDougal & West, 1953; Widdicombe, 1963; Karczewski & Widdicombe, 1969a,b; Colebatch, 1970; Collier & James, 1970; Douglas et al., 1973). The cholinergic agonists, carbachol, pilocarpine, and acetyl- β -methylcholine also constrict airway smooth muscle (Dixon & Brodie, 1903; Widdicombe, 1963; Bouhuys, 1977). Acetylcholine has been shown to produce constriction after bilateral vagotomy although constriction is usually more pronounced in the intact animal. Therefore, at least part of its activity in vivo is direct (Dixon & Brodie, 1903; Karczewski & Widdicombe, 1969b).

In vitro, histamine has been reported to constrict airway smooth muscle in the guinea pig, dog, man, and sometimes rat (Hawkins & Schild, 1951; McDougal & West, 1953; Somlyo & Somlyo, 1970). Tracheal preparations from cats or rabbits have been reported either not to respond to histamine (Akcasu, 1952, 1959; McDougal & West, 1953; Maengwyn-Davies, 1968; Fleisch, Maling & Brodie, 1970; Somlyo & Somlyo, 1970) or to relax (Akcasu, 1952, 1959; Maengwyn-Davies, 1968; Somlyo & Somlyo, 1970; Eyre, 1973; Turker & Ercan, 1976; Chand & Eyre, 1977). Sometimes rabbit trachea has been observed to contract in response to histamine (Somlyo & Somlyo, 1970). Rabbit bronchial tissue contracts in response to histamine (Somlyo & Somlyo, 1970; Chand & Eyre, 1977) but, bronchial tissue from cats or sheep is either unresponsive or relaxes (Eyre, 1969; Persson & Ekman, 1976; Chand & Eyre, 1977). In addition, bronchial tissue from the calf, goat, horse and pig have been reported to contract in response to histamine (Eyre, 1973). These differences, which may partly be explained by anatomical differences among the various species (Akcasu, 1952, 1959; Widdicombe, 1963), are summarized in Table I.

Some of these differences reflect the variation in the nature of the histamine receptors which have been found in the airways of various species. In general, the contractile and relaxant effects of histamine on airway smooth muscle are thought to involve two distinct histamine receptor sub-types (e.g., Chand & Eyre, 1975). Stimulation of histamine H₁-receptors produces contractile effects which can be blocked by the 'classical' antihistaminic drugs such as mepyramine (Ash & Schild, 1966; Chand & Eyre, 1975). In contrast, stimulation of histamine H₂-receptors is thought to result in relaxation of airway smooth muscle and is

TABLE 1

Response of Tracheal and Bronchial Tissues to Histamine

Species	Tracheal Responses			Bronchial Responses		
	Constriction	Relaxation	No response	Constriction	Relaxation	No response
Man	8			2, 3, 5, 9		
Guinea pig	3, 6, 8			9		
Dog	3, 8			9		
Cat		1, 8, 10, 11, 12, 14	3, 4, 8, 10, 12, 15	1, 9, 13	9, 13	1, 9
Rat	3		3, 8, 12			
Sheep				1	13, 14	
Rabbit	4	4, 7, 8, 12, 15	4, 8, 12	4, 7		
Horse				14		
Goat				14		
Calf				14		
Pig				14		

- | | |
|----------------------------|------------------------------------|
| 1. Chand & Eyre, 1977 | 9. Persson & Ekman, 1976 |
| 2. Hawkins & Schild, 1951 | 10. Maengwyn-Davies, 1968 |
| 3. McDougal & West, 1953 | 11. Turker & Ercan, 1976 |
| 4. Somlyo & Somlyo, 1970 | 12. Akcasu, 1952 |
| 5. Guirgis & McNeill, 1969 | 13. Eyre, 1969 |
| 6. Patterson, 1958 | 14. Eyre, 1973 |
| 7. Fleisch & Calkins, 1976 | 15. Fleisch, Maling & Brodie, 1970 |
| 8. Akcasu, 1959 | |

blocked by H₂-antagonists such as burimamide (Black, Duncan, Durant, Ganellin & Parsons, 1972; Chand & Eyre, 1975; Chand & Eyre, 1977; Okpako, Chand & Eyre, 1978). There is some evidence to suggest that stimulation of both H₁ and H₂ receptors is involved with relaxation of airway smooth muscle in the cat (Eyre, 1973).

Stimulation of the histamine H₂-receptors has been correlated with an increase in intracellular levels of cyclic adenosine monophosphate (cAMP) (Lichtenstein & Gillespie, 1973; Mathe, Volicer & Puri, 1974; Mathe, Sohn & Volicer, 1978; Okpako, Chand & Eyre, 1978; Chand & Eyre, 1978). These increases do not appear to involve any effects on phosphodiesterase activity (Mathé, Sohn & Volicer, 1978), and it has been suggested that histamine H₂-receptor activity plays a role in modulating bronchoconstriction induced by stimulation of histamine H₁-receptors (Okpako, Chand & Eyre, 1978).

In addition to species differences, there is also a wide inter-animal variation in sensitivity to histamine (Dale & Laidlaw, 1910; Douglas & Bouhuys, 1969; Douglas et al., 1973). Large differences in sensitivity have also been noted among human subjects (Bouhuys, Jönsson, Lichtneckert, Lindell, Lundgren, Lundin & Ringquist, 1960; Simonsson et al., 1972; Bouhuys, 1977). The variation in sensitivity can largely be attributed to the influences of these factors:

1. Reflex mechanisms which will be discussed later;
2. Effects of anesthetics;
3. Initial tone of the airways;
4. Effects of disease states, and
5. Dose and route of administration.

The direct effects of histamine in vivo have been reported to produce decreases in C_L due to constriction of small airways (Collier, Holgate, Schachter & Shorley, 1960; Karczewski & Widdicombe, 1969a,b; Colebatch, 1970; Mills & Widdicombe, 1970) to approximately the 400 μ size (Nadel et al., 1967). However, others have reported direct effects on large and small airways (Drazen & Austen, 1974). The differences between these observations may be explained by the techniques used. Drazen & Austen (1974) used unanesthetized guinea pigs in which measurements, particularly of C_L , can be distorted by changes secondary to alterations in the pattern of breathing (Mead, 1961; Mills & Widdicombe, 1970). The others used animals anesthetized with urethane, chloralose or pentobarbital. Histamine responses are known to be affected by the anesthetic used (Douglas & Bouhuys, 1969; Dennis & Douglas, 1970; Douglas et al., 1972, 1973; Jackson & Richards, 1977). Urethane and pentobarbital protected the lungs from the effects of histamine and atropine or hexamethonium provided less protection against histamine responses after urethane (Douglas & Bouhuys, 1969). Also Jackson & Richards (1977) reported that a vagal component in the histamine response could be detected when chloralose was used as the anesthetic but not when pentobarbital was used.

It has been suggested that the balance between parasympathomimetic (constricting) and β -adrenergic (dilating) impulses determines the normal tone of airway smooth muscle and hence its sensitivity to histamine (Widdicombe, 1963; Douglas et al., 1973). The influence of autonomic drugs on the response to histamine can therefore be explained in terms of how they shift the initial balance. In general, those

drugs which tend to promote β -adrenergic tone lessen the response to histamine, while those drugs which promote parasympathomimetic tone potentiate responses to histamine (Bouhuys, 1977). β -Adrenergic blocking agents potentiate histamine-induced constrictions (Collier & James, 1967; McCulloch, Proctor & Rand, 1967; Bouhuys, Dennis, & Douglas, 1969), particularly in animals with initial low sensitivity to histamine and therefore may apparently decrease interanimal variation (Collier & James, 1967; Bouhuys, Dennis & Douglas, 1969; Douglas & Bouhuys, 1969; Douglas et al., 1973; Burden, Parkes & Gardiner, 1971).

Parasympathomimetic drugs such as eserine also potentiate the effects of histamine on the airways (Dixon & Brodie, 1903; Hawkins & Schild, 1951; Bouhuys, 1977). Atropine or other parasympathomimetic blocking agents decrease the response to histamine. This effect will be discussed in more detail when reflex constriction is considered.

Exaggerated responses to histamine may occur in patients with conditions such as asthma (e.g. Bouhuys, 1960; Colebatch, 1970; Douglas et al., 1973; Popa, Douglas & Bouhuys, 1973; Bouhuys, 1977). Damaged epithelial tissue (Nadel, 1977), increased autonomic reflexes (Widdicombe, 1963; Douglas et al., 1973; Orehek, Gayrard, Smith, Grimaud & Charpin, 1977), decreased cAMP response to histamine (Mathé, Sohn & Volicer, 1978) and possible pre-existent obstruction or differences in muscle thickness (Orehek et al.) have all been suggested as possible factors determining the increased responsiveness of these individuals.

To some extent, the histamine response also varies according to the dose and route of administration. Injection of small doses into the bronchial circulation tends to cause a localized response in the large

airways, whereas injection into the pulmonary circulation produces the expected effects on the smaller airways (Nadel, 1965; DeKock et al., 1966). Inhalation of histamine droplets $\leq 5 \mu$ affected both C_L and R_L , but particularly C_L (Nadel et al., 1967; Bouhuys, Dennis & Douglas, 1969; Douglas & Bouhuys, 1969).

Another agonist which produces a direct bronchoconstriction is 5-hydroxytryptamine (5-HT). Constriction of the large airways which was unaffected by atropine or vagotomy was noted after 5-HT was injected into the bronchial arteries of cats (Nadel, 1965). Using different routes of administration, other workers have concluded that 5-HT also causes some constriction of smaller airways (Holgate & Warner, 1960; James, 1969).

Thus the parasympathomimetics, histamine and 5-HT have all been shown to produce a direct constriction of airway smooth muscle. The response to histamine has been shown to be subject to marked species differences, and to vary according to the initial state of the airways and the route of administration. Reflex constriction is also an important determinant of airway smooth muscle tone and will be considered next.

C. BRONCHOCONSTRICTION INVOLVING REFLEXES

Airway smooth muscle tone is affected by several reflexes (see Figure 2). Stimulation of irritant or 'cough' receptors, located in the trachea and large bronchi (Miller, 1937; Nadel, 1965) results in reflex constriction; both the afferent and efferent pathways of this reflex involve vagal fibers (Nadel, 1965; Mills, Sellick & Widdicombe,

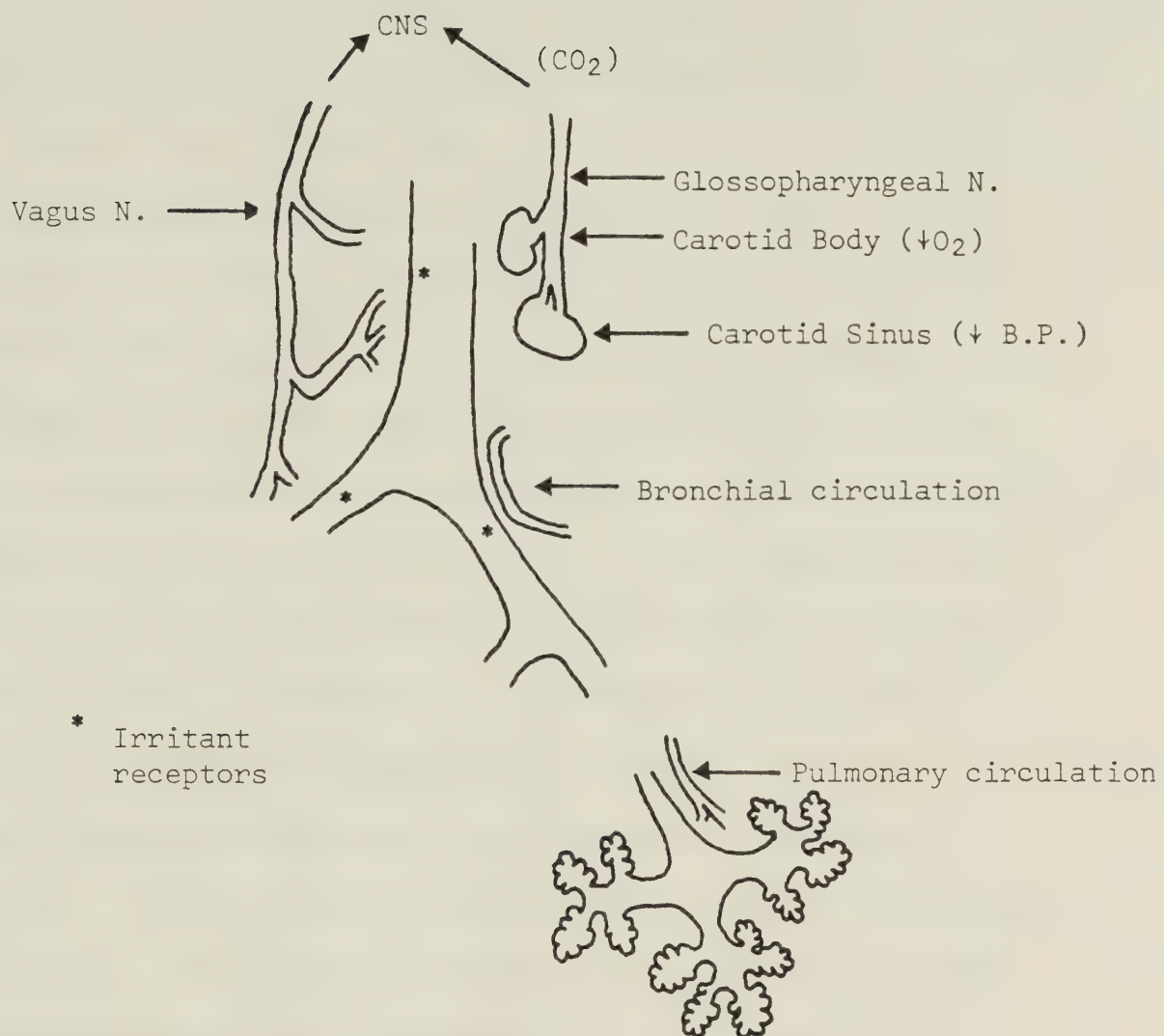


FIGURE 2. Airway reflexes

1969). Hering-Breuer or pulmonary stretch receptors are located throughout the respiratory tract and are involved in two reflexes (Guyton, 1971). When the lungs are stretched, the Hering-Breuer receptors cause a depression of the respiratory center and inhibit inspiration (Berger, Mitchell & Severinghaus, 1977). When the lung tissue is no longer stretched, the receptors become inactive and lung inflation or dilation occurs. The afferent pathways of these reflexes are vagal (Nadel, 1965; Guyton, 1971).

Reflex constriction occurs in response to stimulation of chemoreceptors in the carotid body by decreased pO_2 . Also, stimulation of baroreceptors in the carotid sinus by reductions in blood pressure may produce a slight reflex constriction. The afferent pathways of both these reflexes are mediated by the glossopharyngeal nerves (Nadel & Widdicombe, 1962; Nadel, 1965; Nadel et al., 1967). Hypercapnia has been reported to affect central chemoreceptors and produce reflex constriction of the airways mediated by the vagus nerves (Nadel & Widdicombe, 1962; Nadel, 1965; Nadel et al., 1967).

Reflex constriction induced by hypercapnia may be modified by direct effects. Greene & Widdicombe (1966) reported a direct constricting action of CO_2 . In contrast, a direct relaxant effect of CO_2 has sometimes been observed and may be related to its effects on pH (e.g. Duckles, Rayner & Nadel, 1974).

Histamine has also been reported to produce reflex constriction of the airways (Nadel, 1965; Nadel et al., 1967; Collier, 1968; James, 1969; Mills & Widdicombe, 1970; Douglas et al., 1973). The reflex action of histamine is mediated by the vagus nerve and contributes to the increase

in R_L observed after histamine administration (Nadel, 1965; DeKock et al., 1966; Nadel et al., 1967; Karczewski & Widdicombe, 1969a,b; Mills & Widdicombe, 1970). Therefore, vagotomy, pretreatment with atropine, or pretreatment with hexamethonium have been suggested as possible methods of decreasing the variation of the response to histamine (Douglas & Bouhuys, 1969; Douglas et al., 1973).

Reflex constriction mediated by efferent vagal fibers has four major characteristics:

1. It can be blocked by vagotomy or atropine;
2. It is associated with increased vagal activity to the bronchi;
3. It causes large increases in R_L with little or no effect on C_L , thus producing a prolonged emptying time, and
4. It causes a decrease in tracheal volume (Nadel, 1965; Nadel et al., 1967).

These changes can be explained by assuming constriction of the large airways which are known to be densely innervated by vagal efferent fibers (Dixon & Brodie, 1903; Miller, 1937; Widdicombe, 1963; Nadel, 1965).

In contrast, directly acting agents injected into the pulmonary vasculature cause a bronchoconstriction which is characterized by the following:

1. A decrease in C_L as large or larger than the increase in R_L ;
2. An increase in tracheal volume, and
3. A decreased or unchanged emptying time (Nadel, 1965).

It should also be noted that pretreatment with non-steroidal anti-inflammatory drugs (NSAID) has been observed to modify responses to parasympathomimetics, histamine and 5-HT. Frey (1977) reported an

increase in sensitivity to 5-HT in cats pretreated with ASA. Orehek, Douglas, Lewis & Bouhuys (1973) reported a decrease in response to small doses of histamine or acetylcholine after tissues were pretreated with either ASA or indomethacin, whereas responses to larger doses of both drugs were increased. Similar results were noted when 5-HT, Ba^{++} or K^+ were used as agonists (Bouhuys, 1977). These results may be partially explained by increased release of histamine or increased formation and release of thromboxanes, particularly thromboxane B_2 , in animals treated with NSAID (Engineer, Niederhauser, Piper & Sirois, 1978).

Alternatively, Orehek, Douglas, Lewis & Bouhuys (1973) suggested that these results may be explained by assuming that the sensitivity of the airways to various prostaglandins depends on the tone of the smooth muscle. They proposed that constricted muscles were more sensitive to the relaxant actions of the prostaglandins of the E-type than to the constricting action of prostaglandin $F_{2\alpha}$, and that the reverse was true for dilated or relaxed airway smooth muscle. Both types of prostaglandins are released during the constriction of airway smooth muscle (Orehek et al., 1973; Bouhuys, 1977).

The agonists reviewed thus far (parasympathomimetics, histamine and 5-HT) all produce constriction of airway smooth muscle by direct, reflex, or a combination of direct and reflex, actions. In contrast, agonists which stimulate the β_2 -adrenergic receptors in airway smooth muscle produce marked bronchodilation. These agonists will be discussed in the next section.

D. BRONCHODILATION

One possible mechanism of inducing smooth muscle relaxation involves the raising of cyclic (cAMP) levels within the cell (Lundholm, Mohme-Lundholm & Svedmyr, 1966; Robison, Butcher & Sutherland, 1967; Kukovetz & Pösch, 1970; Bär, 1974; Pösch & Umfahrer, 1976). This may be accomplished by activating the enzyme adenylyl cyclase (AC) which is involved in the synthesis of cAMP or by inhibiting the enzyme phosphodiesterase (PDE) which breaks down cAMP (Robison, Butcher & Sutherland, 1967; Sutherland, Robison & Butcher, 1968). The beta-adrenergic agonists and methylxanthine derivatives are thought to induce bronchodilation by stimulating AC and inhibiting PDE, respectively (Butcher & Sutherland, 1962; Robison, Butcher & Sutherland, 1967; Sutherland, Robison & Butcher, 1968; Farmer & Farmer, 1976; Kreutner & Sherwood, 1977).

Isoproterenol (INA), a beta-adrenergic agonist, is a potent bronchorelaxant (Hawkins & Schild, 1951; McDougal & West, 1953; Collier et al., 1960; Robison, Butcher & Sutherland, 1967; Bouhuys, 1970). Muscle relaxation induced by INA has been correlated with increases in cAMP (Lundholm, Mohme-Lundholm & Svedmyr, 1966; Sutherland, Robison & Butcher, 1968). The bronchodilator effects of aminophylline or theophylline (Castillo & de Beer, 1947; Hawkins & Schild, 1951; Butcher & Sutherland, 1962) have also been correlated with changes in cAMP levels (Sutherland, Robison & Butcher, 1968; Kukovetz & Pösch, 1970; Kreutner & Sherwood, 1977). While INA is equally effective in relaxing tone induced by either acetylcholine or histamine (McDougal & West, 1953), aminophylline has a more selective effect, being more active against histamine-induced tone (McDougal & West, 1953). It is possible that

other phosphodiesterase inhibitors may show different actions and selectivities, as PDE exists in several molecular forms and the isozymes might respond differently to individual inhibitors (Weiss & Hait, 1977).

As the β -sympathomimetics and the methylxanthines have separate and distinct mechanisms of action, additive or synergistic actions should be possible (Lichtenstein & Margolis, 1968; Pösch & Umfahrer, 1976; DeTarnowsky & Green, 1977; Kreutner & Sherwood, 1977; Dyson & Campbell, 1978; Marlin, Hartnet, Berend & Hackett, 1978). DeTarnowsky & Green (1977) could detect no potentiation of INA-induced increases in cAMP by theophylline in embryonic or newborn chick liver, but Lichtenstein & Margolis (1968) reported synergistic effects with INA and theophylline when measuring histamine release from leucocytes in vitro. Pösch & Umfahrer (1976) have reported an enhancement of orciprenaline-induced relaxations of K^+ -induced contractions of guinea pig colon by aminophylline, papaverine, caffeine and other PDE inhibitors. Also, when interaction between theophylline and the β_2 -adrenergic agonists, rimiterol and salmefamol, was examined in vivo (Dyson & Campbell, 1978; Marlin et al., 1978), additive, but not synergistic, therapeutic effects were reported when theophylline was combined with rimiterol (Dyson & Campbell, 1978). When theophylline was combined with salmefamol, synergy was observed in producing tremor in patients' hands, but only additive therapeutic effects were observed (Marlin et al., 1978). It therefore appears that additive effects are more likely than synergistic effects when the drugs are used in therapeutic concentrations.

E. METHODOLOGY REVIEW

The actions of drugs on the respiratory system have been evaluated using a variety of preparations, in vitro and in vivo (e.g. Bouhuys, 1970). The guinea pig has been the species most often used for evaluating the actions of agents on airway smooth muscle (Mills & Widdicombe, 1970). Castillo & de Beer (1947) reported the use of tracheal chain or bronchial preparations for the evaluation of the activity of bronchodilators. McDougal & West (1953) compared the responses of human bronchial preparations with the responses of tracheal ring preparations from several species using histamine, acetylcholine and various bronchodilators. They concluded that the relative potencies of bronchodilators were similar in human and guinea pig preparations. Their work confirmed that done by Hawkins & Schild (1951). In addition to reacting in a manner similar to human tissue, the guinea pig tracheal preparation has the advantage of developing inherent tone, thus relaxants can be examined without inducing tone with an agonist such as histamine (Castillo & de Beer, 1947).

The effects of substances on pulmonary ventilation have been determined in vivo with techniques that measure either total lung resistance to inflation or the individual changes in C_L and R_L . Methods used to measure changes in total lung resistance are based on the technique described by Konzett & Rössler (1940). This type of method is referred to as an air overflow technique and will be described in more detail in Chapter 2 under methods. Air overflow experiments provide no information about the mechanisms underlying the changes in total resistance although,

in general terms, these changes are thought to primarily reflect constriction of small airways (Holgate & Warner, 1960; Collier & James, 1967; Collier, 1968). Another disadvantage of air overflow experiments is that tone must be induced before bronchodilating activity can be detected (James, 1968). In contrast, the methods developed to measure changes in R_L and C_L can detect bronchodilation without initial constriction.

To determine the values of C_L and R_L , three parameters must be measured:

1. The rate of volume change or air flow rate ($\dot{V}$),
2. The total volume of air that has flowed in the airways (V), and
3. The changes in lung pressure associated with changes in $\dot{V}$ and V .

The V is easily measured by directing the airflow through a calibrated pneumotachograph connected to a suitable pressure transducer. Integration of the signal from the pneumotachograph provides the means for measuring V as $\int \dot{V} dt = V$. Proper heating of the pneumotachograph is required to minimize errors in volume measurements resulting from air at room temperature and humidity being warmed and humidified within the respiratory tract. Alternatively, volume can be determined by plethysmography. The subject is confined in a gas-tight chamber or plethysmograph and changes in volume are measured as changes in gas volume or pressure within the chamber (Nunn, 1977).

Pressure changes associated with lung distention, or transpulmonary pressure (P_{TP}), have been the most difficult of the parameters to measure (Mead, 1961). Basically, four approaches have been used to measure changes in P_{TP} :

1. Interrupter valves,
2. Intrapleural catheterization,
3. Intraesophageal measurements, and
4. Intratracheal measurements.

The first technique consists of having the patient make vital capacity expirations through a valve system using either single or multiple interruptions. After a sudden interruption of flow, the pressure at the valve has been shown to correspond with the P_{Tp} immediately before interruption (Shephard, 1959). This method requires patient cooperation and the results are affected by changes in muscular power. Also, the method is not practical for animal experiments.

The second method involves placing a cannula into the pleural space and connecting its free end to a differential pressure transducer which is also connected to the airway opening. Usually, a small degree of pneumothorax is introduced to prevent clogging of the cannula (Mead, 1961).

In the third method, intraesophageal pressure is measured. Changes in P_{Tp} are similar to changes in pressure within the esophagus (Mead & Whittenberger, 1953; Mead, 1961). Although the absolute level of intraesophageal pressure may be slightly higher than pleural pressure, changes at both sites were equal if the esophagus was relaxed, unless high rates of respiration were used (Fry, Stead, Ebert, Lubin & Wells, 1952; Mead & Whittenberger, 1953; Mead & Gaensler, 1959). Posture also affects esophageal pressures; when in the supine position the overlying musculature can compress the esophagus and reduce accuracy (Mead & Gaensler, 1959; Mead, 1961; Nunn, 1977).

The fourth method involves the determination of pressure within the trachea. For subjects in a supine posture, measurement of intra-tracheal pressure provides a better estimation of P_{TP} than does intra-esophageal pressure. This value is obtained by connecting a pressure transducer to a sidearm of a tracheal cannula and is closely related to pressure within the lungs when positive pressure ventilation is used (Greene & Widdicombe, 1966; James, 1969).

Pulmonary ventilation can be described in terms of $\dot{V}$, V and ΔP , and the relationship between these parameters is defined by the following equation:

$$\Delta P_L = f(V) + f(\dot{V}) + f(\ddot{V}) \quad (1)$$

where ΔP_L is the total pressure change between the airway opening and pleural surface, that is, transpleural pressure (P_{TP}); $f(V)$ defines a function of the volume and therefore involves the component of the pressure change associated with changes in compliance or P_{CL} ; $f(\dot{V})$ defines a function of the rate of change of V and involves pressure changes associated with changes in flow resistance or conductance, P_{RL} ; and $f(\ddot{V})$ defines a function related to inertial forces of the air and tissues (Mead, 1961). The inertial forces are slight, less than 5% of the total pressure change during heavy breathing, and can be ignored (Mead & Whittenberger, 1953; Brody, DuBois, Nisell & Engelberg, 1956; Mead, 1961; Dennis, Douglas, Casby, Stolwijk & Bouhuys, 1969; Derks & Lisart, 1977). Therefore, equation 1 can be rewritten as:

$$\Delta P_L = \Delta P_{CL} + \Delta P_{RL} \quad (2)$$

At points of zero airflow, resistance forces are assumed to be zero and only elastic forces are present (Mead & Whittenberger, 1953; Mead, 1961; Dennis et al., 1969) and thus equation 1 reduces to $\Delta P_L = \Delta P_{CL}$ at these times. Points of zero airflow occur at the beginning and end of each respiration cycle, that is, at the end of inspiration and again at the end of expiration, making these times convenient for the measurement of lung compliance. At the end of inspiration, the pressure can be defined as:

$$P_L = P_{Li} = P_0 + K\Delta V \quad (3)$$

where P_0 is the pressure at the beginning of inspiration, P_{Li} is the pressure at the end of inspiration, ΔV is the amount of air inspired, and K is a term dependent on lung elasticity; K is further defined as $1/C_L$. At the end of expiration, the pressure, P_{Le} , returns to the value of P_0 . The change in pressure is obtained by subtracting:

$$\Delta P_L = \Delta P_{Li} - \Delta P_{Le} = K\Delta V = 1/C_L (\Delta V) \quad (4)$$

Solving equation 4 for C_L results in

$$C_L = \Delta V / \Delta P_L \quad (5)$$

Thus, if values for changes in volume and pressure are measured and plotted on a graph of ΔV vs ΔP , C_L can be calculated as the slope of the line (Figure 3a). In practice, non-linearity can arise from several factors (Mead, 1961). At very high lung volumes, smaller changes in volume per unit of pressure occur. Also, if the pressure is increased for several respiratory cycles, lung elastance is reduced and compliance is increased. In anesthetized animals, C_L tends to fall with time but this can be reversed by near maximum inflations (Mead, 1961).

To determine R_L , equation 2 is solved for R_L :

$$\Delta P_{RL} = \Delta P_L - \Delta P_{CL} = f(\dot{V}) \quad (6)$$

As resistance changes are a function of flow, and the variables $\dot{V}$ and P_L are measured, it is convenient to examine the function by plotting $\dot{V}$ vs ΔP_L . A plot of this type for a complete respiratory cycle has the form of a closed loop (Figure 3b). There are two points in the loop corresponding to the end of inspiration and the end of expiration at which $\dot{V} = 0$. Between these points, as there is no airflow, the pressure change reflects the component of total pressure change due to elastic forces or compliance changes. This component is eliminated from the overall function by subtracting ΔP_{CL} from the function. When this is done, the plot becomes linear and the slope of the line is defined as conductance, the inverse of R_L . R_L is therefore the reciprocal of the slope of $\Delta P_L / \Delta V$.

For a more detailed treatment of these methods, see Mead & Whittenberger (1953) or Mead (1961). The rationale for investigating the actions of diazoxide on airway smooth muscle is provided in the next section.

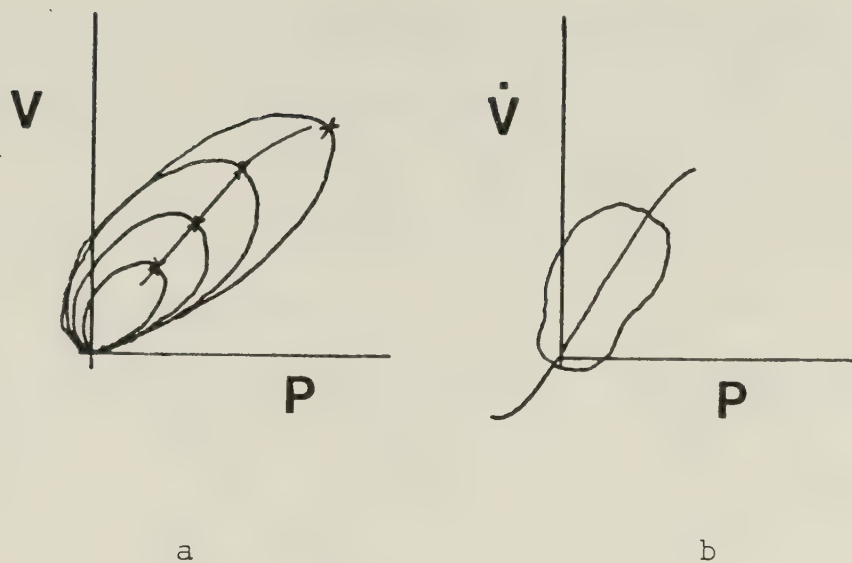


FIGURE 3. Respiration Loops

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- a) Volume-pressure loops used to determine lung compliance.
X's mark peak of loop obtained with each volume.
 - b) Flow-pressure loop before (closed loop) and after (curvilinear plot) subtraction of the compliance component of pressure.

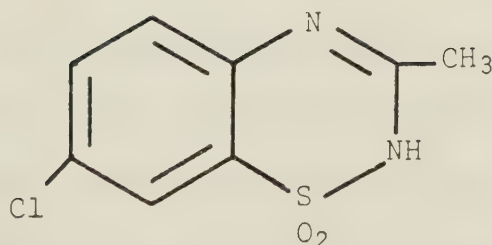


FIGURE 4. Diazoxide

F. DIAZOXIDE

Diazoxide is a benzothiadiazine derivative (Figure 4) used intravenously in the treatment of hypertensive crises (Rubin, Roth, Taylor & Rosenkilde, 1962; Rubin, Taylor & Roth, 1968; Drugs, 1971). Usually, one rapid intravenous injection of 300 mg (20 ml) produces an immediate marked reduction in blood pressure which lasts several hours. Transient hyperglycemia often accompanies single doses and, after repeated dosing, may become persistent (Loubatières, Mariani & Alric, 1968; Drugs, 1971). When given orally, diazoxide produced a decreased hypotensive effect but an increased and prolonged hyperglycemia (Rubin, Taylor & Roth, 1968). For these reasons, diazoxide has also been used to treat hypoglycemic disorders (Weyer, 1968).

Although diazoxide was shown to release noradrenaline from extra-adrenal tissues, the hypotensive action was not affected by prior administration of propranolol (Loubatières, Mariani & Alric, 1968; Drugs, 1971). Pretreatment with drugs such as atropine, hexamethonium and phenoxybenzamine also failed to affect the hypotensive response (Rubin et al., 1962). It has therefore been suggested that diazoxide produces its hypotensive effects by a direct relaxation of vascular smooth muscle, particularly in the arterioles (Rubin, 1963; McNeill, Barnes, Davis & Hook, 1969). In contrast, release of catecholamines was involved in the hyperglycemic response to diazoxide (Loubatières, Mariani & Alric, 1968; Tabachnick & Gulbenkian, 1968; Symchowicz & Korduba, 1968; Senft, 1968; Porte, 1968; Marks & Samols, 1968). The ability of diazoxide to decrease insulin secretion and to inhibit PDE also contribute to the hyperglycemic response (Loubatières, Mariani &

Alric, 1968; Drugs, 1971; Tabachnick & Gulbenkian, 1968; Senft, 1968; Fajans, Floyd, Thiffault, Knopf, Harrison & Conn, 1968; Porte, 1968; Seltzer & Crout, 1968; Bleicher, Chowdhury, Podolsky, Fleischman & Goldner, 1968). Using enzyme preparations in vitro, Moore (1968) concluded that diazoxide possessed 0.28 the potency of theophylline as an inhibitor of PDE.

The ability of diazoxide to inhibit PDE and release catecholamines suggests that it might have some bronchodilating activity. A review of the literature revealed that some patients commented on "easier breathing" after receiving diazoxide (Just & Stein, 1969). This study was therefore undertaken to determine if diazoxide had any actions on respiratory smooth muscle and, if so, to try and determine the mechanisms involved in those actions.

MATERIALS AND METHODS

The methods used for these investigations have been divided into three groups which will be discussed separately. The three categories which will be discussed are the following:

1. In vitro procedures,
2. Air overflow measurements, and
3. Determinations of R_L and C_L

A list of the chemicals, drugs and solutions used in this study is provided in Appendix I.

A. ISOLATED TISSUE PREPARATIONS

Experiments in vitro were used in preliminary studies to determine if diazoxide (D) produced changes in the tone of airway smooth muscle. The effects of aminophylline (A) and isoproterenol (INA) were also determined and compared with those of diazoxide. A and INA were chosen because they are used clinically as bronchodilators and because they are thought to relax smooth muscle by increasing the levels of cAMP (see Introduction, p. 19).

The preparation chosen for the majority of these experiments was the guinea pig tracheal chain (Castillo & de Beer, 1947; Akcasu, 1952; Edinburgh Staff, 1968). Human bronchial and tracheal preparations were also tested. Unfortunately, tissues for such experiments could not be obtained routinely and only a very few experiments could be done.

B. GUINEA PIG TRACHEAL CHAIN PREPARATION

A carbon dioxide atmosphere was prepared by placing dry ice in the bottom of a dessicator. Random bred, male guinea pigs weighing 450 - 1000 g were stunned by placing them in the dessicator. Once stunned, the radial artery was severed and the animal bled to death. The trachea was dissected, cleared of any adhering tissue and cut transversely between segments of cartilage to yield four or five multi-ringed pieces (see Figure 5). Four pieces were stitched together such that the sections of smooth muscle were on alternating sides. To complete the chain, each piece was cut through the cartilage at a point opposite from the smooth muscle.

Two chains, each from a separate animal, were tied to a glass tissue holder and mounted in a 10 ml tissue bath containing Krebs solution heated to 37°C and gassed with 95% O₂ / 5% CO₂. The free ends of the tissues were attached to Grass Model FT03C Force Displacement Transducers connected to a Grass Model 7D Polygraph. The tissues were placed under an initial tension of 300 mg and allowed to equilibrate for one hour. During that time, inherent tone developed and the tension developed by the muscle increased.

After the tissues had equilibrated, drugs were injected into the bath according to a scheme shown in Figure 6. All drugs except diazoxide were added to the bath in volumes $\leq$ 0.5 ml. Because of its low solubility in Krebs solution (see Appendix 1) it was sometimes necessary to inject a volume of 2 ml of a solution containing diazoxide. In these cases, an equal amount of Krebs solution was removed from the bath before the

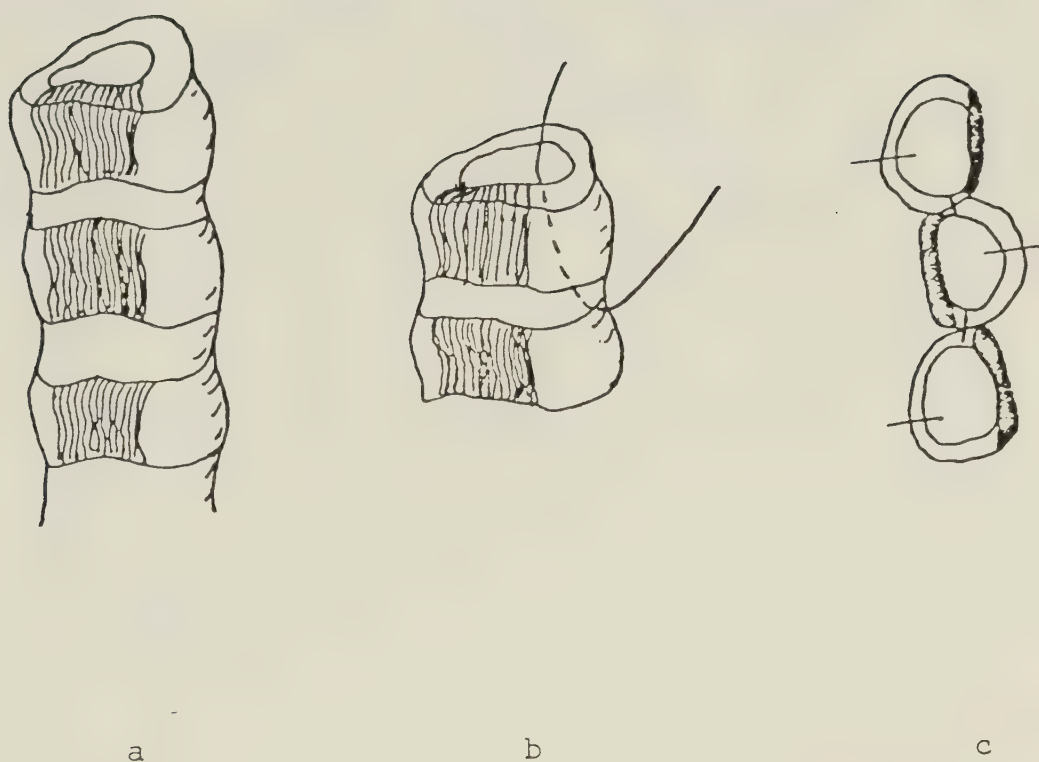


FIGURE 5. Preparation of tracheal chains

- a) The trachea was cleared of any adhering tissue.
- b) The trachea was cut into four or five multi-ringed pieces.
- c) Four pieces were stitched together to form chain.

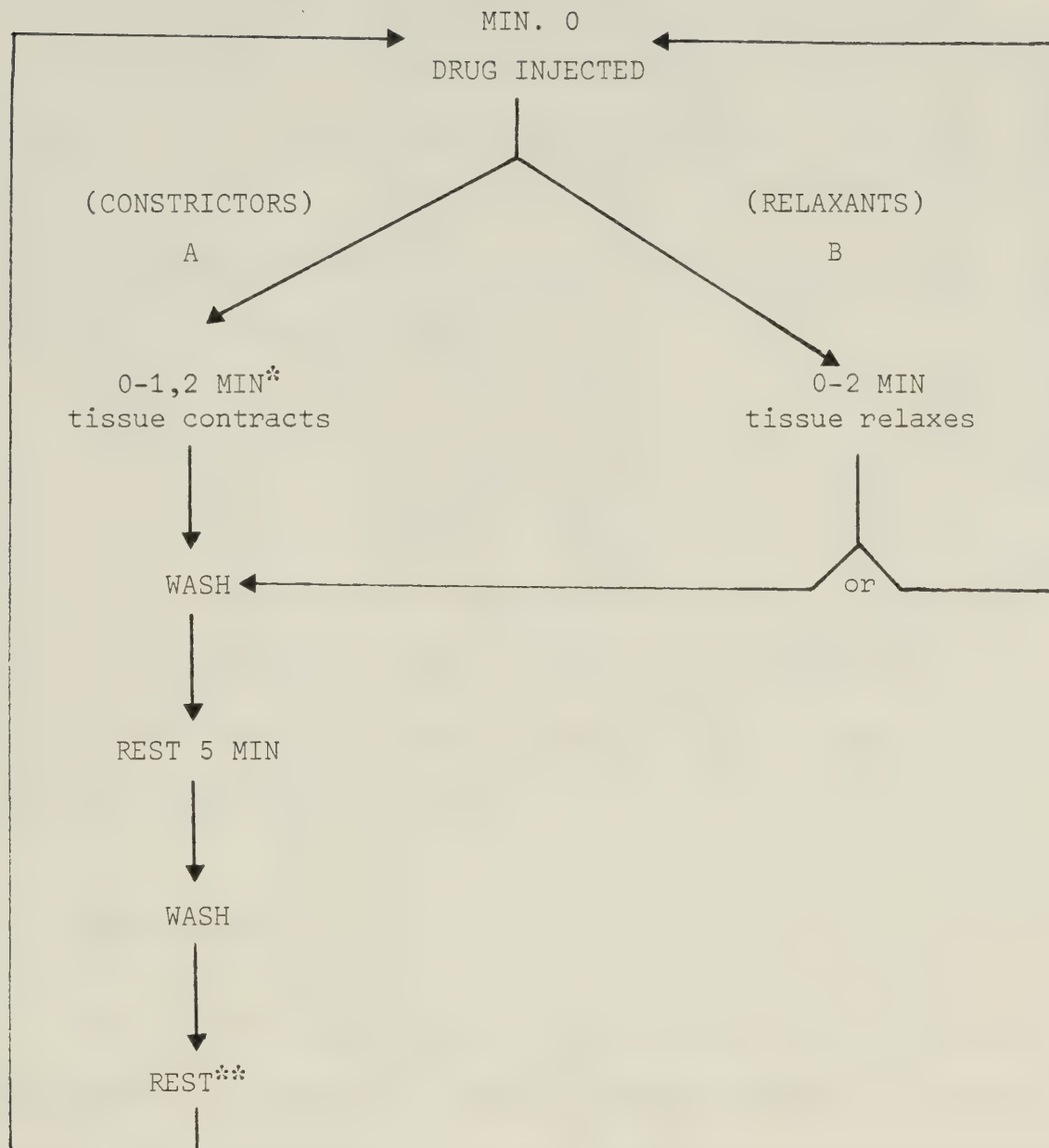


FIGURE 6. Schedules used for Tracheal Chain Experiments

* 1 min if constrictor alone, 2 min if relaxant and constrictor.

** rest 10 min if constrictor alone; rest 15 min if relaxant and constrictor.

diazoxide was added. The tissues were washed by overflow with 25-30 ml of heated Krebs solution pumped from the bottom of the bath.

A single dose of histamine was repeated according to Scheme A, Figure 6, until tissue responses were uniform and then a dose-response curve to histamine was obtained. A second dose-response curve to histamine was obtained in the presence of one or more relaxant drugs according to Scheme B, Figure 6.

The maximum response of the tissue was determined using 11.8 μ g of acetyl- β -methylcholine or acetylcholine injected into the bath according to Scheme A, Figure 6. Combinations of relaxants were tested after responses to the respective drugs in the combination had been tested separately. When the influence of propranolol on relaxant activity was tested, propranolol was added to the Krebs solution and was present continuously.

C. HUMAN TISSUES

Human tissues were obtained during surgery and placed in chilled, oxygenated Krebs solution. Strips of smooth muscle were cleared and mounted in a tissue bath in a manner similar to the guinea pig tracheal chains. The tissues were tested in a manner similar to the tracheal preparations except that cumulative dose-response curves were obtained. In addition, the tissues were allowed to rest longer in between cycles as they were much slower to recover.

D. MEASUREMENTS OF AIR OVERFLOW

As diazoxide appeared to be an effective bronchodilator in vitro (see Results) its activity in vivo was evaluated using an air overflow technique. A and INA were again chosen as model bronchodilators for these experiments.

Random bred, male guinea pigs weighing between 250-700 gm were anesthetized with urethane, 1.5 mg/kg, given intraperitoneally. Some animals were also given an additional injection of 2.5-5 mg of sodium pentobarbital. The trachea was cannulated and connected to a Harvard Small Animal Respiration Pump (see Figure 7). The lungs were inflated with the pump set at 25 RPM and 10 ml stroke. A constant inspiratory pressure was maintained by connecting the inspiratory side of the pump to a 10 cm column of water. Air not entering the lungs escaped through the water column and the flow was measured by a Fleisch pneumotachograph No. 0.707 connected to a Grass Volumetric Pressure Transducer Model P75 A. As the lungs became more difficult to inflate, the amount of air escaping through the water column increased. Increases in the overflow were recorded with a Hewlett-Packard 7754A recorder with Model 8805 carrier amplifiers. Since the responses were too small for convenient measurements using this system, they were further amplified using a Hewlett-Packard Model 680 M Strip Chart Recorder connected to the output of the 8805 carrier amplifier.

The jugular vein was cannulated and all drugs used in the experiments were administered through the cannula in volumes ≤ 0.5 ml. After injection, the cannula was flushed with an additional 0.2 ml of normal saline. Sodium pentobarbital, 2.5 mg, was injected at the start of each

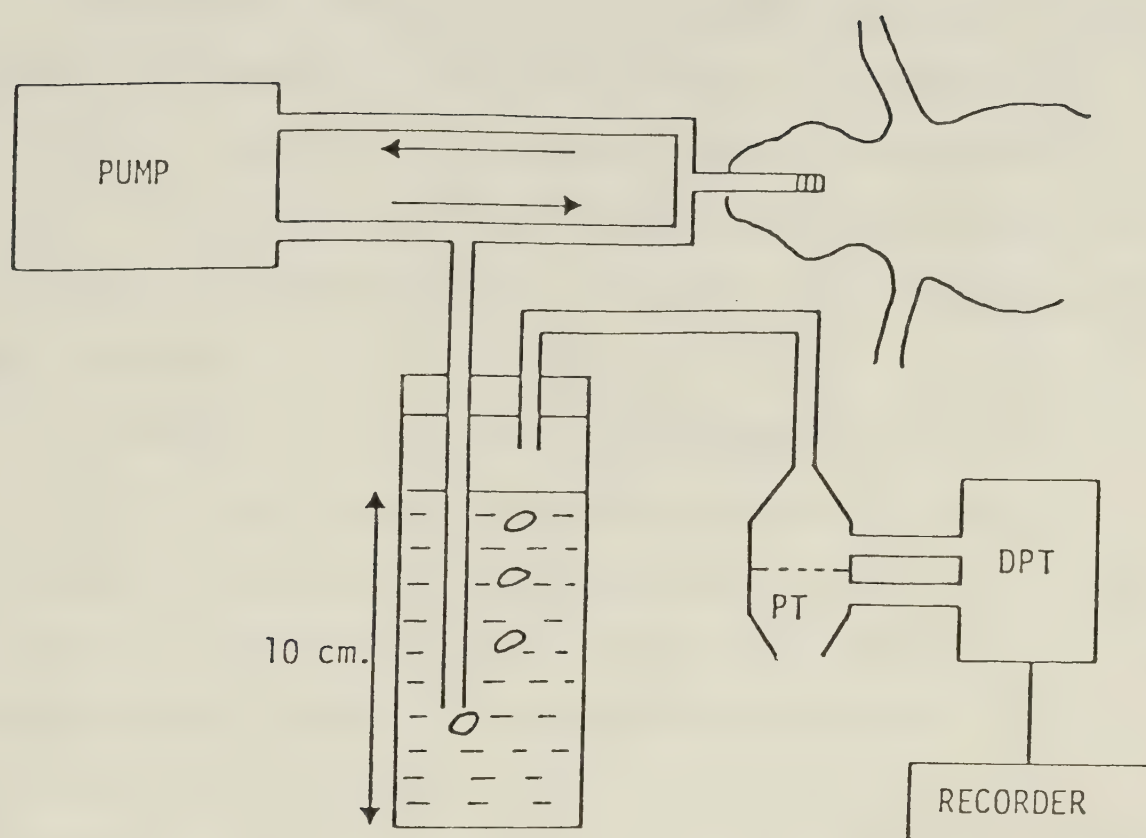


FIGURE 7. Air overflow Apparatus.

PT = Pneumotachograph

DPT = Differential pressure transducer

experiment to eliminate spontaneous breathing. After bronchoconstricting drugs were administered, recovery was sometimes aided by occluding the overflow tube (to increase V) as suggested by Collier et al. (1960).

The effects of histamine, diazoxide, aminophylline and INA were examined in each animal. Three doses of each dilator were tested and the influence of the order of administration was also examined.

In some experiments, the carotid artery was cannulated and the blood pressure was monitored using a Statham pressure transducer Model P23Dd connected to the Hewlett-Packard recorder.

One disadvantage of this type of experiment is the necessity of inducing some degree of bronchoconstriction in order to detect any bronchodilating activity. Histamine was used to induce tone in these experiments. A second disadvantage is that only changes in total lung resistance can be measured and the respective contributions of changes in compliance or flow resistance to the total change could not be distinguished. As changes in total lung resistance had been observed after diazoxide was administered (see Chapter 3, Results) further experiments were performed in vivo to measure specifically the effects of diazoxide on C_L and R_L .

E. MEASUREMENTS OF C_L AND R_L

Values for C_L and R_L were determined using a method similar to that described by Mead and Whittenberger (1953). Random bred, male guinea pigs weighing 287.5-1048 g were anesthetized with urethane, 1.75 g/kg, given intraperitoneally. This dose is larger than that used for the air overflow experiments, however, sodium pentobarbital was

never added. The trachea was cannulated and connected to the Harvard Small Animal Respiration Pump set to a constant rate of 20 RPM in a manner similar to that used in the air overflow experiments (see Figure 8). In these experiments, the pneumotachograph was placed between the pump and the tracheal cannula. The side-arm of the cannula was used to monitor intratracheal pressure and was connected to a Grass Volumetric Pressure Transducer Model PT5 A. The signal from this transducer (P_T) provided an estimate of the parameter P_{TP} . The pneumotachograph was connected to a Statham P23BB transducer and provided a measure of $\dot{V}$. Appropriate pairs of signals (V and P_T or $\dot{V}$ and P_T) were initially displayed simultaneously in order to ensure that measurements were not distorted by phase lags between signals.

I. DETERMINATION OF R_L

For the determination of R_L , the $\dot{V}$ and P_T signals were displayed on the y-axis and x-axis, respectively, of a Tektronix 5113 oscilloscope equipped with Tektronix 5A22N differential amplifiers and a 5B12N Dual Time Base. The dual time base was used as a differential amplifier, thus the oscilloscope became an x/y plotter. In order to remove the compliance component from this plot, a pressure signal equal to P_{CL} was electronically subtracted from the P_T signal. The angle, θ , of the resulting line was measured and its slope calculated as $\tan \theta$. R_L was then determined by calculating the reciprocal of the slope, $\cot \theta$ (Figure 3). Resistance measurements were made at 7.5 ml ventilation volumes for animals larger than 450 g and at 6.5 ml for smaller animals. When not measuring C_L , the animals were ventilated at these volumes.

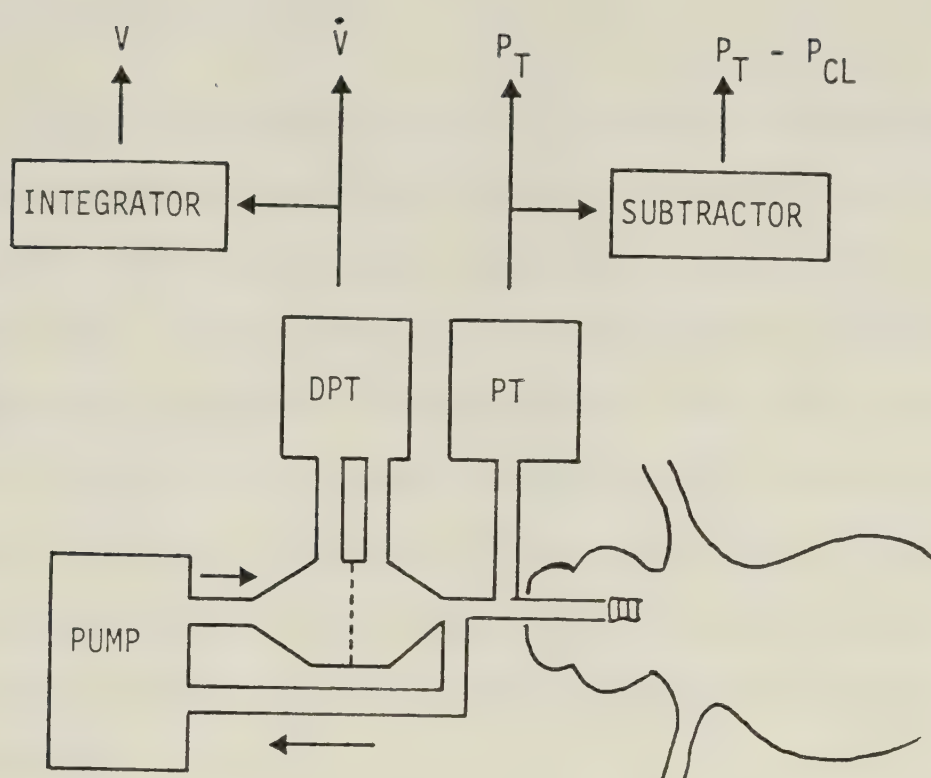


FIGURE 8. Apparatus for measurements of C_L and R_L .

V = Volume

$\dot{V}$ = Flow rate

PT = Pressure transducer

DPT = Differential pressure transducer

P_{RL} = Flow resistance component of pressure

P_{CL} = Compliance component of pressure

P_T = Tracheal pressure

II. DETERMINATION OF C_L

For the determination of C_L , volume and pressure measurements were recorded at three pump volumes, depending on the size of the animal. For animals larger than 450 g, volumes of 5, 7.5 and 10 ml were used. Volumes of 5, 6.5 and 9 ml were used for smaller animals. The volume measurement was obtained by electrical integration of the $\dot{V}$ signal from the pneumotachograph. The values for V and P_T were plotted on the y-axis and x-axis of a graph, respectively. The best straight line through these values was determined using linear regression analysis, and C_L was calculated as the slope of that line.

Circuit diagrams for the integrator and subtractor units are provided in Appendix 2.

The jugular vein was cannulated and drugs administered as in the air overflow experiments. Pancuronium bromide, 0.1 mg/kg, was administered at the start of each experiment to paralyze the respiratory muscles and eliminate the effects of changes in the pattern of breathing. Two animals required an additional dose of this drug during the course of the experiment; the second dose was reduced to 0.05 mg/kg. It was assumed that the compliance of the chest wall remained constant and that changes in C_L therefore reflected changes in lung compliance.

The carotid artery was cannulated and blood pressure was monitored with a Statham P23BB transducer. Blood samples were taken at the start and just prior to the administration of the bronchodilator to be tested from some of the animals. Samples were obtained from either the carotid artery or the jugular vein. A 1 ml syringe, rinsed with and containing

0.1 ml heparinized saline was attached to the cannula and 0.5-1 ml of blood withdrawn. The syringe was immediately capped until analysis. All samples were tested within approximately 5 min of sampling. The samples were analyzed for pH, $p\text{CO}_2$ and $p\text{O}_2$ values on an Instrumentation Laboratory Micro 13 pH/Blood Gas Analyzer.

Only one bronchodilating agent was tested in each animal. The effects of histamine and 5-HT were examined before and after administration of the relaxant drug to be tested. The effects of atropine, vagotomy, mepyramine, indomethacin and ASA on the initial increase in total lung resistance observed after diazoxide administration (see Results) were investigated. The effects of imidazole, a PDE activator, on diazoxide activity were investigated. All experiments were performed in triplicate.

F. STATISTICAL METHODS

The Student's t-test, standard error of the mean difference, analysis of variance, and Duncan's Multiple Range Test were used as described by Snedecor (1967).

RESULTS

The results from the experiments in vitro, the air overflow experiments and the measurements of C_L and R_L will be presented separately.

A. GUINEA PIG TRACHEAL CHAINS

I. EFFECTS ON INHERENT TONE

Slow, moderate reductions in tone were noted after the addition of diazoxide, 1.3×10^{-4} M, aminophylline, 0.22×10^{-4} M, or INA, 6.3×10^{-9} M, to the bath (Figure 9, a-c). The potency ratio of aminophylline and INA, based on these doses, is 3500 which is in reasonable agreement with the ratio of 10,000 reported by Hawkins & Schild (1951) for equiactive doses of aminophylline and INA.

When the dose of diazoxide was doubled, no additional relaxation was observed. Higher doses of diazoxide were not tested because of its low solubility in Krebs solution (see Chapter 2, Appendix I). Also, 1.3×10^{-4} M diazoxide was already equivalent to a bath concentration three times greater than the therapeutic plasma concentration (10 μ g/ml) of diazoxide (Drugs, 1971).

Aminophylline, 0.22×10^{-4} M, was judged a less effective relaxant than diazoxide, 1.3×10^{-4} M, using the standard error of the mean difference. However, when the dose of aminophylline was doubled, more relaxation was produced and the differences between aminophylline and diazoxide were no longer statistically significant. Higher concentrations of aminophylline would have corresponded to toxic plasma levels and, therefore, were not tested.

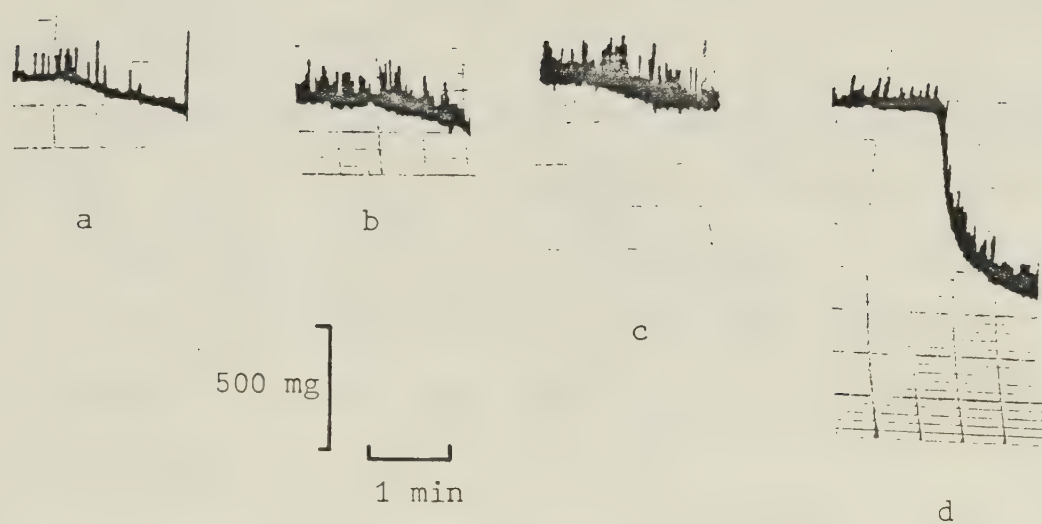


FIGURE 9. Relaxation of inherent tone induced by diazoxide, aminophylline and INA.

-
- a) Diazoxide 1×10^{-4} M
 - b) Aminophylline 0.22×10^{-4} M
 - c) INA 6.93×10^{-9} M
 - d) INA 2.5×10^{-8} M

In contrast to either aminophylline or diazoxide, higher doses of INA produced prompt and marked relaxation (see Figure 9, d). Hawkins & Schild (1951) have also commented on the slow development of relaxation induced by aminophylline as compared to that induced by INA.

Combinations of diazoxide and aminophylline had additive effects (see Figure 10). Combinations of either diazoxide or aminophylline with INA did not significantly alter the response to INA, however, if all three drugs were added to the bath, the response was significantly lower than the response to INA alone (see Figure 11).

As aminophylline (and diazoxide) and INA are thought to raise cAMP levels by different mechanisms (Butcher & Sutherland, 1962; Robison, Butcher & Sutherland, 1967; Sutherland, Robison & Butcher, 1968; Farmer & Farmer, 1976; Kreutner & Sherwood, 1977), the combination of these drugs would have been expected to potentiate the response to INA if relaxation was mediated by cAMP. However, in these experiments, the maximum relaxation was not determined, and it is possible that the maximum had been achieved with INA alone. Also, Nousiainen et al. (1977) have suggested that potentiation of β_2 -agonists is greater and easier to detect than potentiation of INA. Therefore, the possibility of potentiation was also investigated using salbutamol and diazoxide. In these experiments, all responses were expressed as percentages of the maximum. Salbutamol, 5×10^{-9} M, produced a marked relaxation that was potentiated by diazoxide, 1.3×10^{-4} M (see Figure 12).

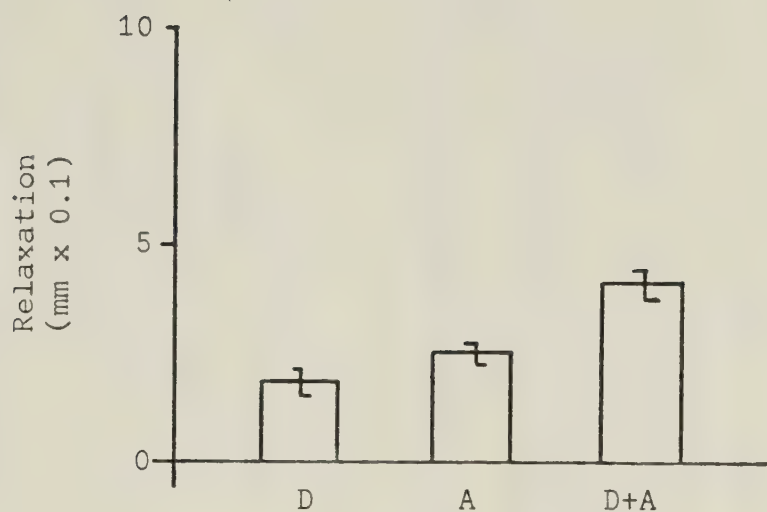


FIGURE 10. Effects of diazoxide and aminophylline on the normal tone of guinea pig tracheal chain preparations.

Bars represent mean values of relaxation obtained from four preparations when diazoxide, 1.3×10^{-4} M (D), and aminophylline, 0.44×10^{-4} M (A), were tested, vertical lines show S.E.

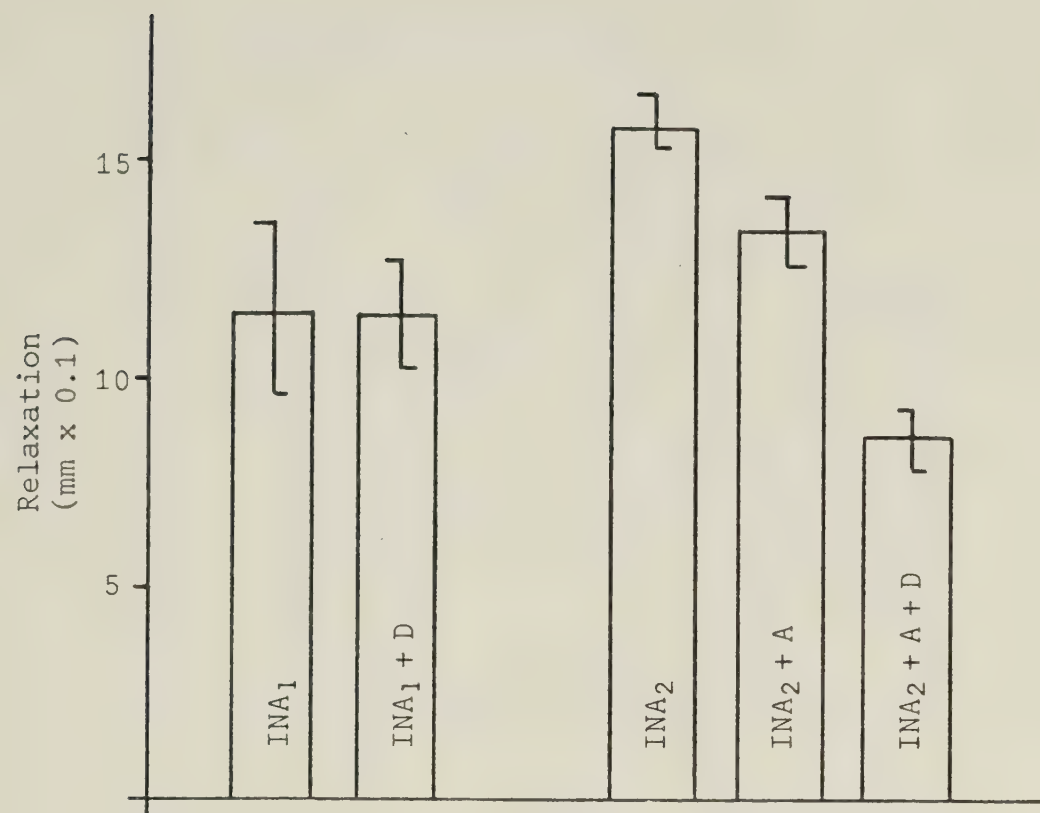


FIGURE 11. Effects of diazoxide and aminophylline on response to INA.

INA₁ = INA 2.5×10^{-8} M

INA₂ = INA 1×10^{-7} M

A = Aminophylline 0.22×10^{-4} M

D = Diazoxide 1.3×10^{-4} M

Bars represent mean values obtained from four tracheal chain preparations. Vertical lines show S.E.

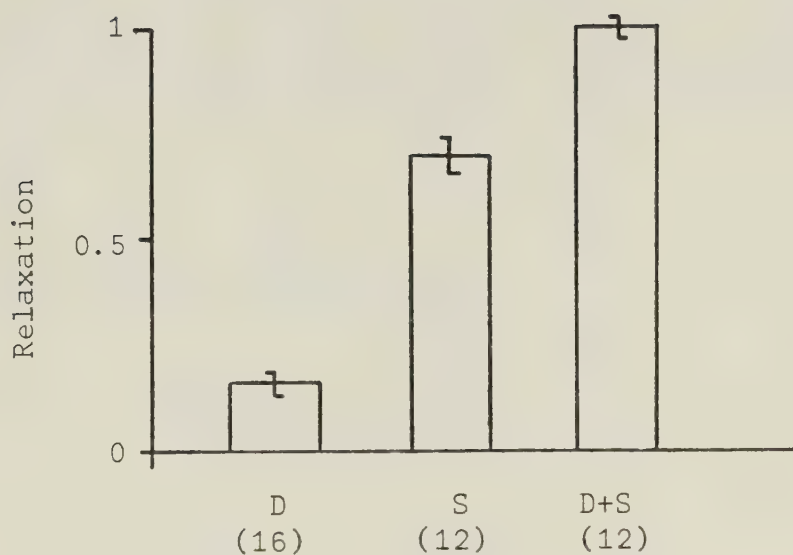


FIGURE 12. Effects of salbutamol and diazoxide on the normal tone of guinea pig tracheal chain preparations.

Bars represent mean value of the fraction of maximal relaxation observed after salbutamol (S), 5×10^{-9} M, and Diazoxide (D), 1.3×10^{-4} M. Vertical bars show S.E. and n is given in parentheses.

Occasionally, a contraction was observed when diazoxide was added to the bath (see Figure 13). The contraction lasted less than one minute and was only noticed in a few tissues. However, when the results of the air overflow experiments were obtained, the traces were re-examined and small contractions were noted in most tissues.

II. EFFECTS ON HISTAMINE-INDUCED TONE

In these experiments, the responses to histamine (2.5×10^{-6} M to 2×10^{-5} M) were obtained before and after the addition of one of the following drugs or drug combinations:

1. Diazoxide, 1.3×10^{-4} M = D
2. Aminophylline, 0.22×10^{-4} M = A
3. INA, 2.5×10^{-8} M = I_1
4. INA, 5×10^{-8} M = I_2
5. INA, 1×10^{-7} M = I_3
6. A + I_1
7. A + I_3
8. D + I_1
9. D + I_2
10. D + I_3
11. A + D + I_1
12. A + D + I_3

All of the drugs blocked the effects of 2.5×10^{-6} M histamine. When the histamine dose was doubled, A, A + INA_1 and A + D + INA_1 were no longer effective. Only

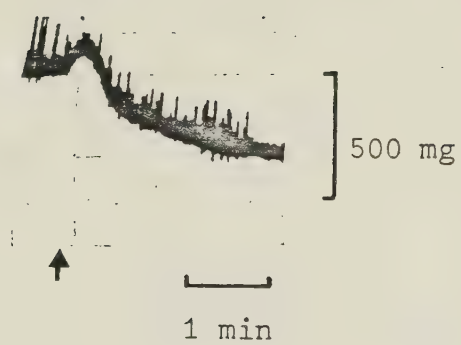


FIGURE 13. Diazoxide-induced contraction.

Diazoxide 1.3×10^{-4} M added at arrow.

combinations of D + INA or A + D + INA₃ effectively inhibited responses to 1×10^{-5} M histamine, and no drug or combination tested inhibited the response to 2×10^{-5} M histamine. These results are illustrated in Figures 14 and 15. The dose ratio of aminophylline and INA in these experiments was 880 which is in good agreement with the 1000-7000 ratio reported by McDougal & West (1953) for equiactive doses.

III. EFFECTS OF PROPRANOLOL

Propranolol, 6.8×10^{-7} M, was used to investigate the possibility that diazoxide was acting by a mechanism which involved stimulation of β -receptors. The effects of diazoxide on normal tone were unaffected by propranolol, whereas the response to INA was abolished (see Figure 16). The effects of diazoxide on histamine-induced tone were also unaffected. Similar results were obtained with aminophylline.

B. HUMAN TISSUE

I. TRACHEAL

One human tracheal ring was obtained and tested at two different tensions. Initially, the ring was mounted in the bath with just enough tension to keep it vertical as suggested by Castillo and de Beer (1947). Diazoxide, 3.25×10^{-4} M, abolished the contractile effect of histamine even when the dose of histamine

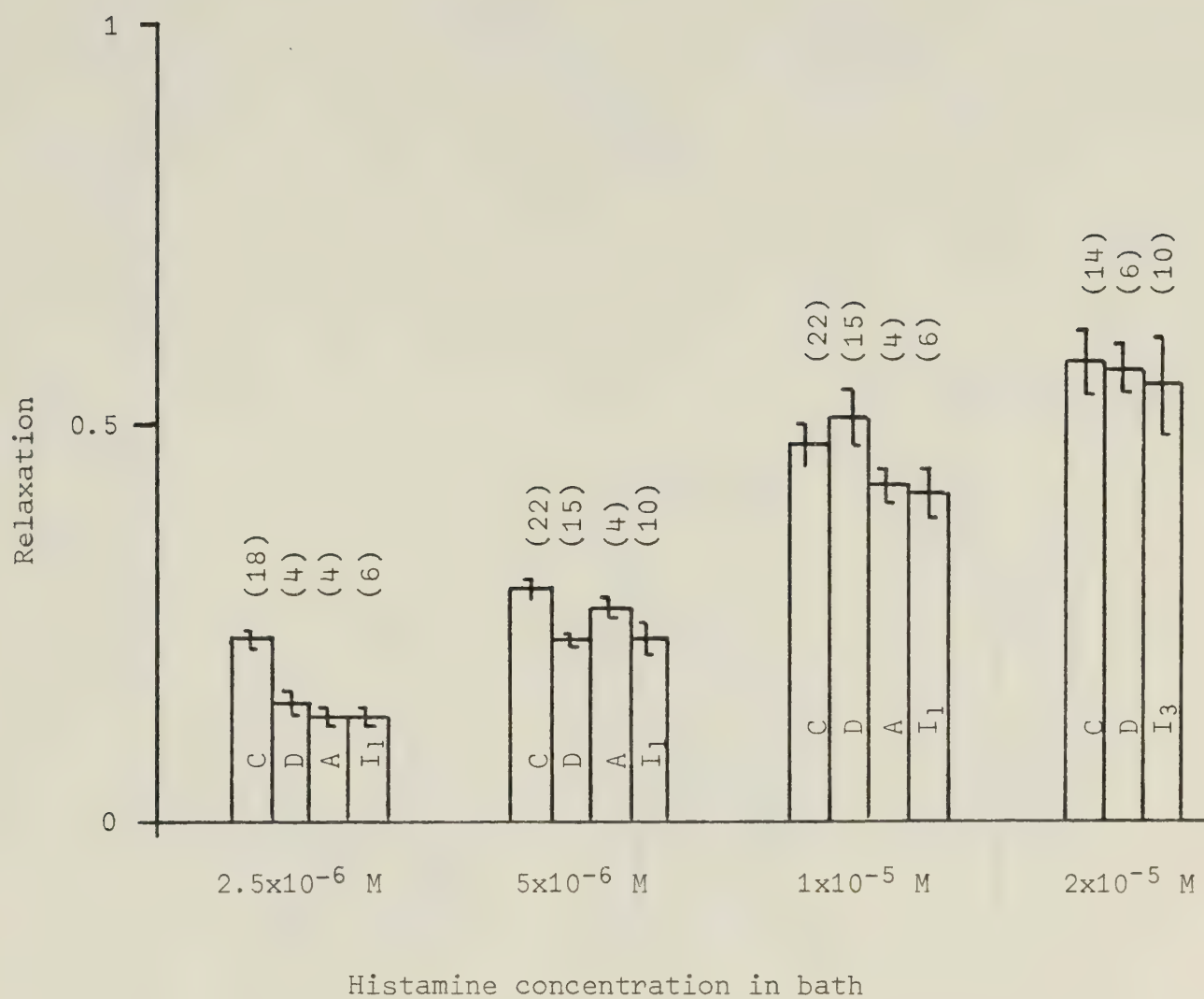


FIGURE 14. Effects of relaxants on responses to histamine.

Bars represent mean response to histamine in control tissues (C) and in tissues treated with diazoxide (D), 1.3×10^{-4} M, aminophylline (A), 0.22×10^{-4} M, and INA, 2.5×10^{-8} M (I₁), or 1×10^{-7} M (I₃). Vertical lines show S.E. and n is given in parentheses.

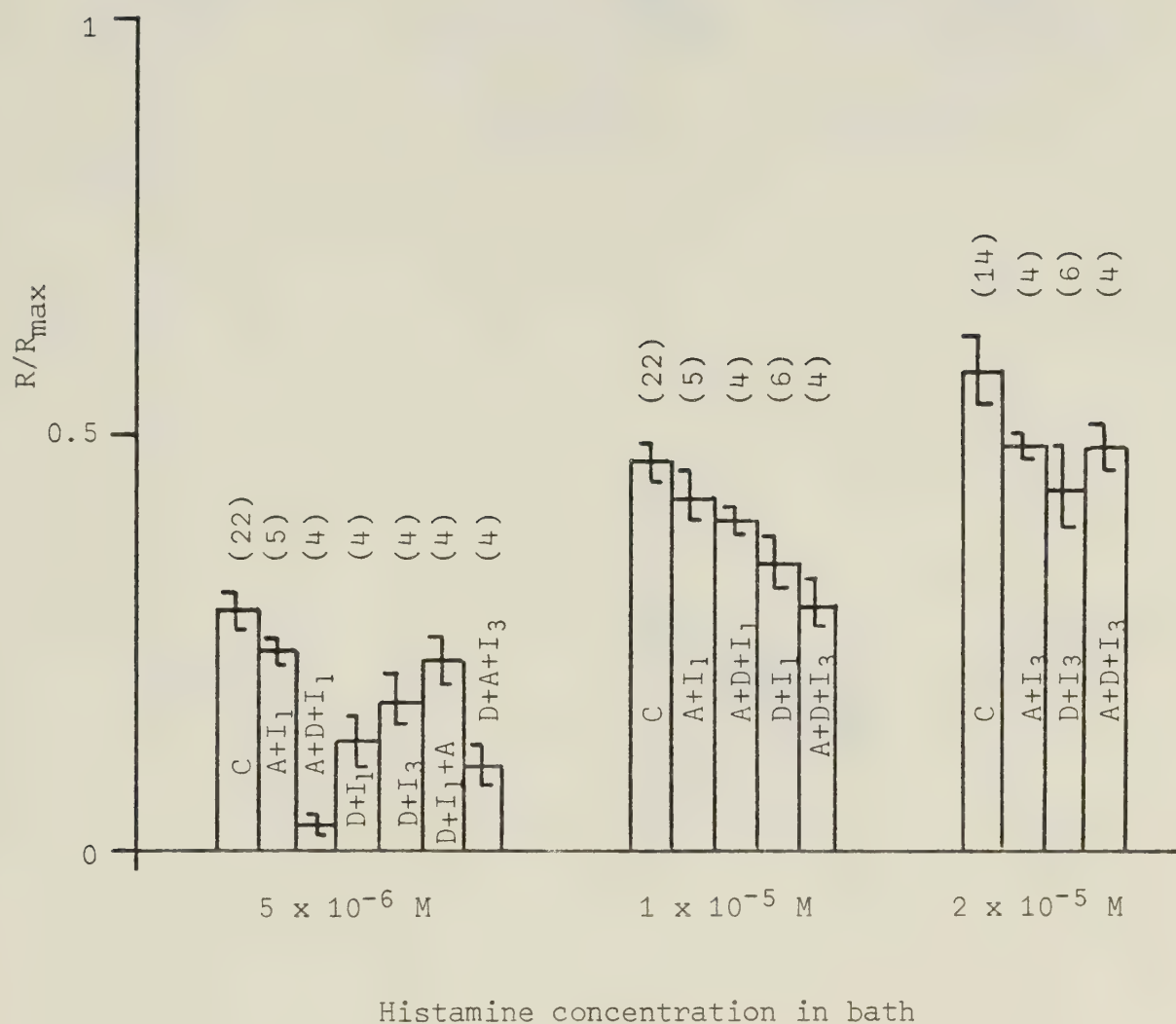


FIGURE 15. Effects of combinations of relaxants on responses to histamine.

Bars represent mean response to histamine in control tissues (C) and tissues treated with some combination of diazoxide 1.3×10^{-4} (D), aminophylline 0.22×10^{-4} (A), INA $2.5 \times 10^{-8} \text{ M}$ (I₁), $5 \times 10^{-8} \text{ M}$ (I₂) or $1 \times 10^{-7} \text{ M}$ (I₃). Vertical lines show S.E. and n is given in parentheses.

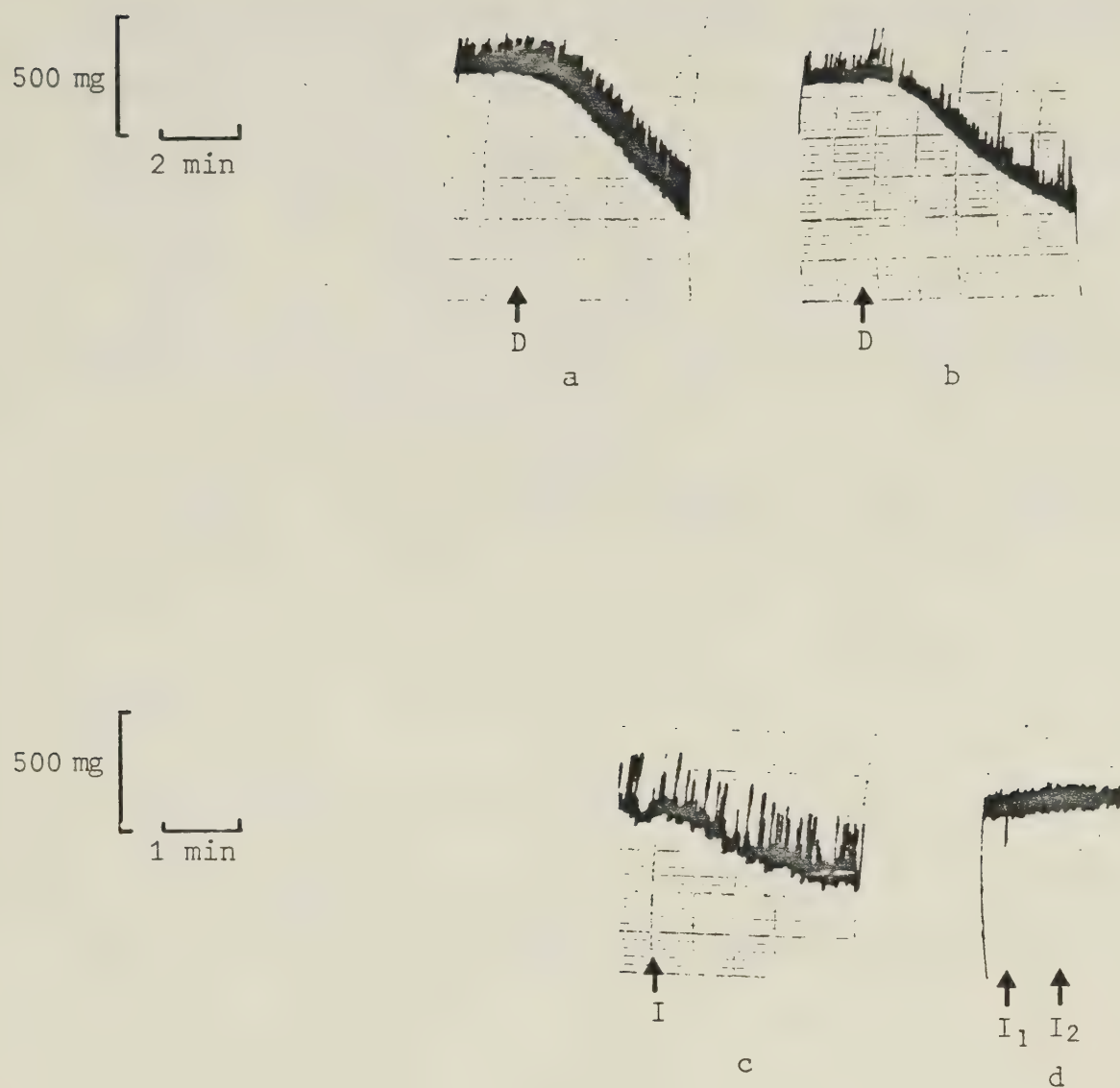


FIGURE 16. Effects of propranolol on diazoxide activity.

-
- a) Response to diazoxide 1.3×10^{-4} M (D) in control tissue.
 - b) Response to D in presence of propranolol 6.8×10^{-7} M.
 - c) Response to INA 1.25×10^{-8} M (I) in control tissue.
 - d) Response to INA 1×10^{-7} M (I_1) and 2×10^{-7} M (I_2) in presence of propranolol 6.8×10^{-7} M.

used was equal to that producing a maximal response before the addition of diazoxide. Reducing the dose of diazoxide to 3.25×10^{-5} M produced effects similar to that seen in the guinea pig tracheal chain preparations.

After the two doses of diazoxide had been tested, tension on the ring was increased by 500 mg. Sensitivity of the preparation to histamine increased and diazoxide, in concentrations of 3.25×10^{-5} M, 6.5×10^{-5} M or 1.3×10^{-4} M, failed to antagonize the response to histamine.

II. BRONCHIAL

Three human bronchial preparations were tested. One preparation failed to develop tone and did not respond to any of the drugs tested. Both of the remaining preparations developed inherent tone which was reduced by diazoxide, 1.3×10^{-4} M, or aminophylline, 0.22×10^{-4} M. Aminophylline, 0.44×10^{-4} M failed to antagonize the response to histamine in either preparation. Diazoxide, 2.6×10^{-4} M, failed to antagonize the effects of histamine in the second preparation, but 1.3×10^{-4} M diazoxide effectively inhibited the contractile effect of histamine in the third. These results are summarized in Table 2.

TABLE 2

Human Bronchial Preparations

	Preparation		
	1	2	3
Developed inherent tone	No	Yes	Yes
Diazoxide decreased inherent tone	-	Yes	Yes
Aminophylline decreased inherent tone	-	Yes	Yes
Histamine caused contraction	No	Yes	Yes
Diazoxide antagonized histamine	-	No	Yes
Aminophylline antagonized histamine	-	No	No

C. AIR OVERFLOW EXPERIMENTS

I. INCREASED RESISTANCE TO INFLATION

Eighteen animals were used in this series of experiments. In each animal, histamine produced a dose-related increase in total lung resistance to inflation. The mean ED_{50} of histamine was $0.44 \mu\text{g}$ (S.E. 0.04). Individual ED_{50} 's ranged from $0.09 \mu\text{g}$ to $0.83 \mu\text{g}$. Typical responses to histamine are shown in Figure 17.

Just as an initial contraction had been observed in the tracheal chain preparations, an initial increase in total lung resistance was observed when diazoxide was first injected in vivo (see Figure 18). The increase was dose-related, could be reversed by occlusion of the overflow tube, and was detected in approximately 70% of the animals. A similar, but milder, increase was observed in about 25% of the animals after injection with aminophylline, although the animal usually recovered within one minute without the occlusion of the overflow tube. An increase in total lung resistance was never noted after administration of INA. This initial increase was investigated more thoroughly in the experiments in which R_L and C_L were measured, and these results will be presented in the section dealing with the measurements of R_L and C_L .

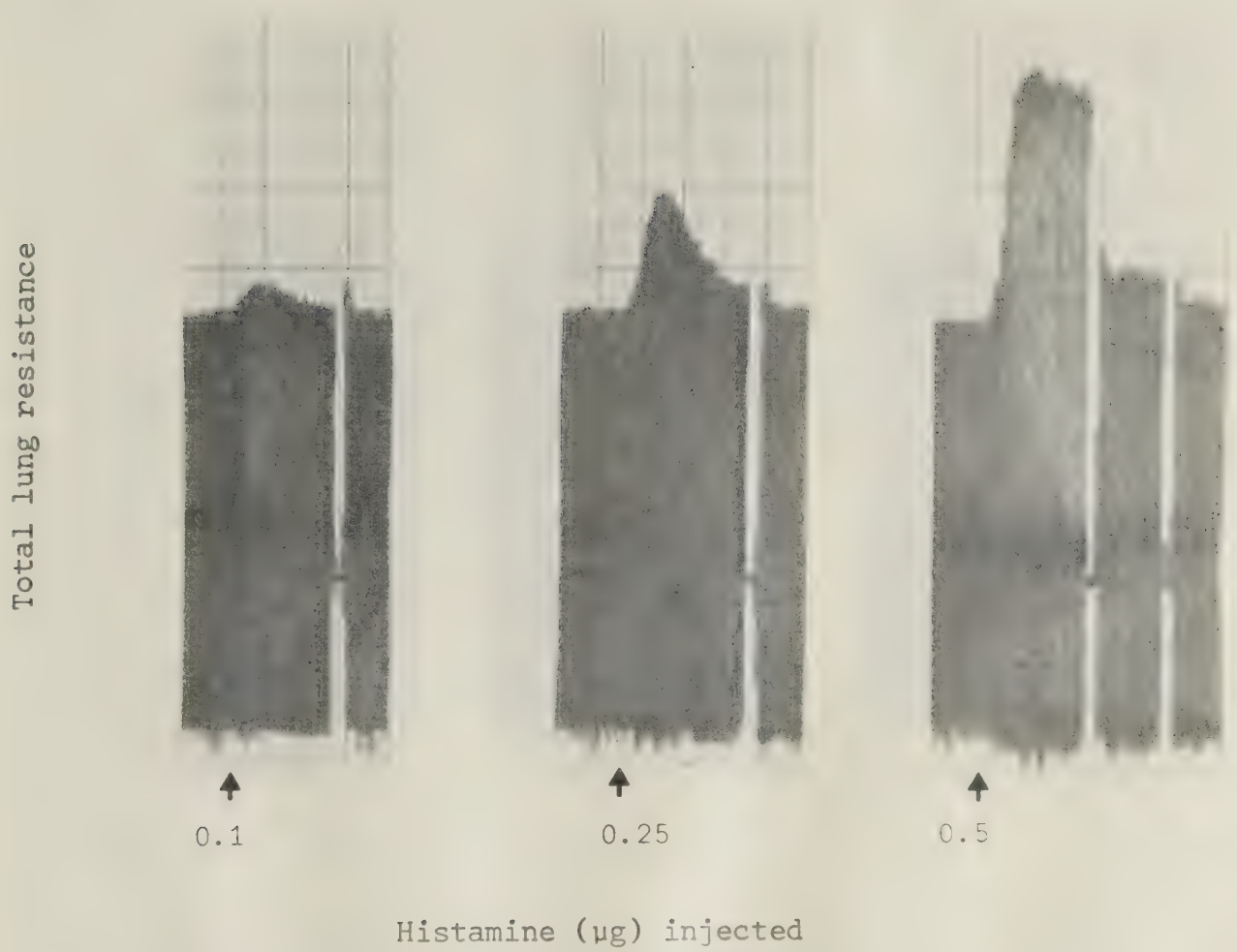
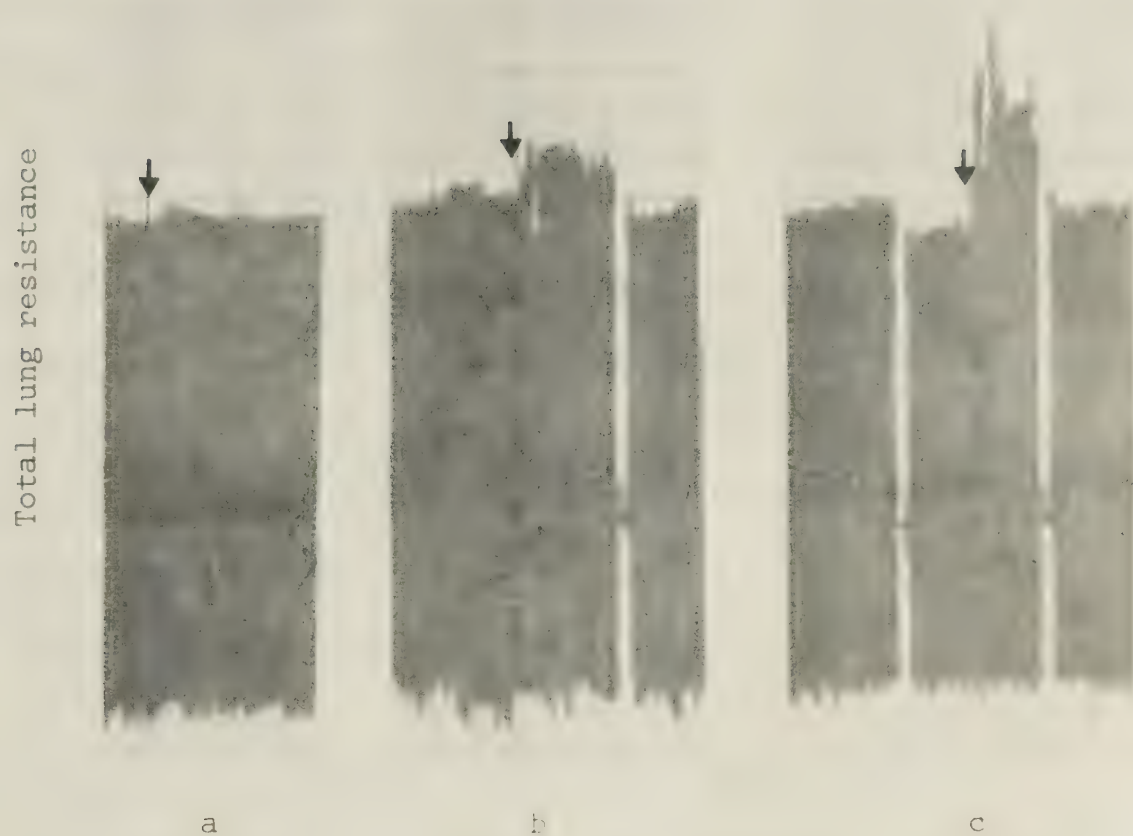


FIGURE 17. Responses to histamine in typical air overflow experiment.



Arrows show time of injection of diazoxide, 10 mg/kg (a), 20 mg/kg (b) and 40 mg/kg (c).

FIGURE 18. Response to diazoxide in air overflow experiment.

II. DECREASED TOTAL LUNG RESISTANCE

Diazoxide, aminophylline or INA effectively inhibited the histamine-induced increases in total lung resistance. These drugs were administered in the following doses:

1. Diazoxide, 10, 20 and 40 mg/kg
2. Aminophylline, 7.5, 15 and 30 mg/kg
3. INA, 0.018, 0.035 and 0.07 μ g/kg.

The data obtained from the first six animals was analyzed using the standard error of the mean difference (Snedecor, 1967). This analysis showed that inhibition of the response to histamine was significant at the 5% level for the following minimum doses tested:

1. Diazoxide, 10 mg/kg
2. Aminophylline, 7.5 mg/kg
3. INA, 0.035 μ g/kg.

In a few animals, 0.018 μ g/kg of INA appeared to inhibit the response to histamine and, therefore, this dose was not eliminated from subsequent experiments. All three drugs blocked histamine in a dose-related manner. Results from typical experiments in which diazoxide or aminophylline was administered are shown in Figures 19 and 20.

The results obtained when D, A, or INA was the first drug tested are summarized in Figure 21. Although their effects were dose-related in individual animals, interanimal variation was large enough that no significant difference was noted among the three doses of each drug. Although it appears that INA was less effective than either D or A, when an analysis of variance

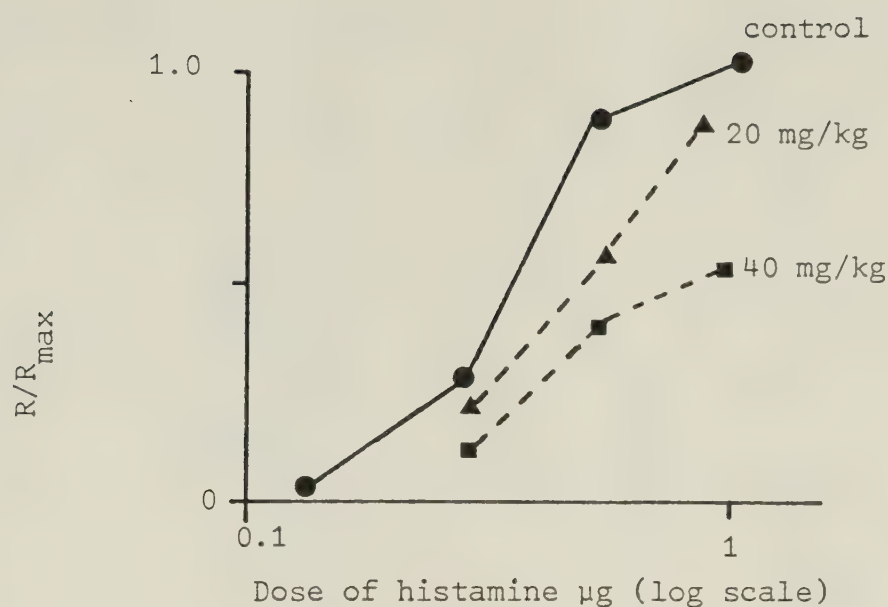


FIGURE 19. Inhibition of response to histamine by diazoxide.

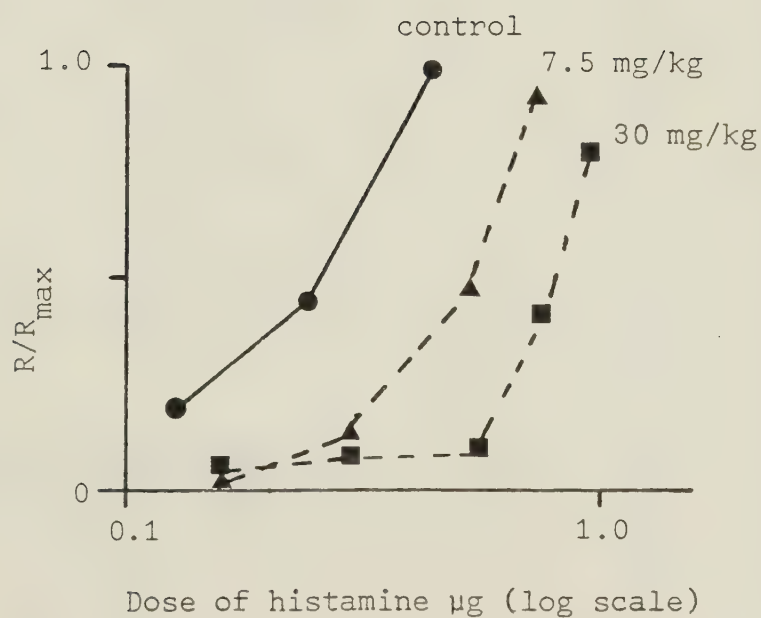


FIGURE 20. Inhibition of response to histamine by aminophylline.

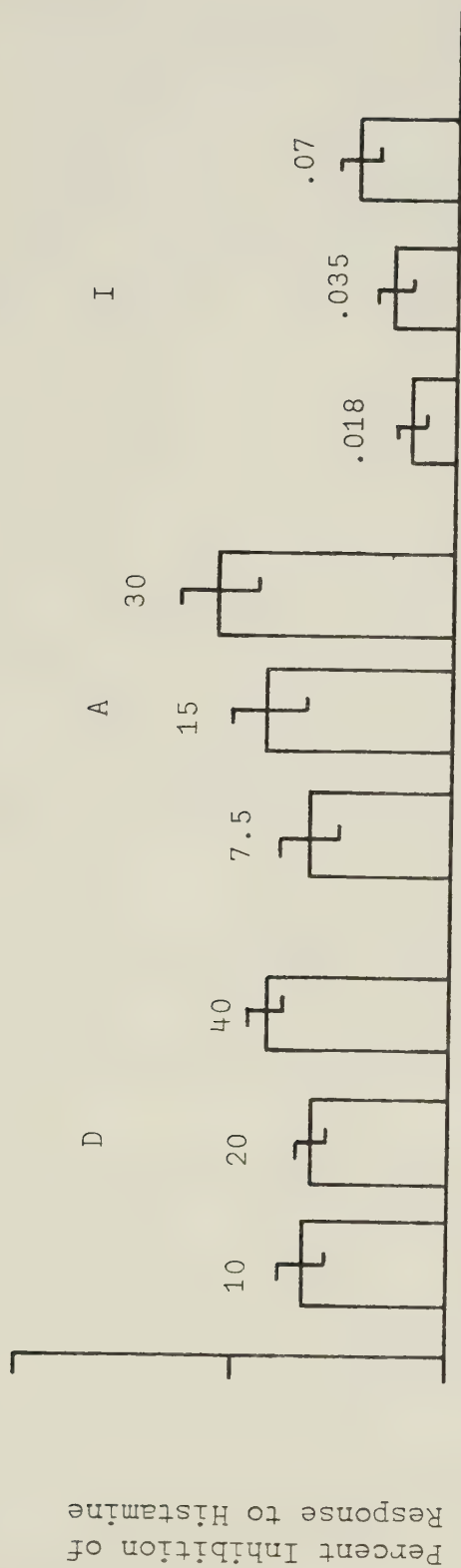


FIGURE 21. Effects of diazoxide, aminophylline and INA on response to histamine.

Bars represent mean inhibition of response to histamine in six animals after administration of diazoxide (D), 10, 20 and 40 mg/kg; aminophylline (A), 7.5, 15 and 30 mg/kg; or INA (I), 0.018, 0.035 or 0.07 μ g/kg. Vertical lines show S.E.

(Snedecor, 1967) was done in which each drug and dose was classified as a separate treatment, no significant differences among the treatments was found. However, the order in which the drugs were administered did produce a statistically significant difference. The difference was further analyzed using Duncan's Multiple Range Test (Snedecor, 1967). At the 1% level, the following influence of the order of administration was found:

$$DIA \equiv DIA \equiv ADI > IDA \equiv IAD \equiv AID$$

A summary of the analysis of variance is shown in Table 3.

If only data obtained from the first two relaxants administered was analyzed, a statistically significant (5% level) difference was noted between the drugs used. Again using Duncan's Multiple Range Test to examine the differences, it was determined that:

$$A(7.5, 15, 30 \text{ mg/kg}) \equiv D(10, 20, 40 \text{ mg/kg}) > INA(0.018, 0.035, 0.07 \text{ } \mu\text{g/kg})$$

A summary of this analysis of variance is provided in Table 4.

D. MEASUREMENTS OF R_L AND C_L

Examples of the resistance loops obtained in these experiments are shown in Figure 22-24. Figure 22 shows resistance loops which were obtained with 5 (inner), 7.5 and 10 (outer) ml pump volumes. The loops appear elongated due to a mechanical artifact associated with the pump. Figure 23 shows a single loop before (a) and after (b) the compliance component was subtracted. The large, distorted U-shaped portion in Figure 23b is the part due to the mechanical artifact. Figure 24 shows

TABLE 3
Analysis of Variance for Air Overflow Experiments

Source of Variation	DF	SS	MS	F	Values of F From F Table	
					5%	1%
Drugs	8	3.7	0.463	1.65	2.18	2.99
Order	5	8.39	1.678	5.99*	2.45	3.51
D x O	40	11.21	0.280	1.14	1.51	1.79
Error	108	26.41	0.245			
Total	161	49.71				

* Statistically significant

TABLE 4
Analysis of Variance with Two Relaxants Administered

Source of Variation	DF	SS	MS	F	Values of F From F Table	
					5%	1%
Drugs	8	7.65	0.96	10.67*	2.18	2.99
Order	5	5.75	1.15	12.78*	2.45	3.51
D x O	40	3.54	0.09	0.3	1.61	1.96
Error	54	16.01	0.30			
Total	107	32.95				

* Statistically significant



FIGURE 22. Resistance loops obtained with 5 (inner), 7.5 and 10 (outer) ml pump volumes.

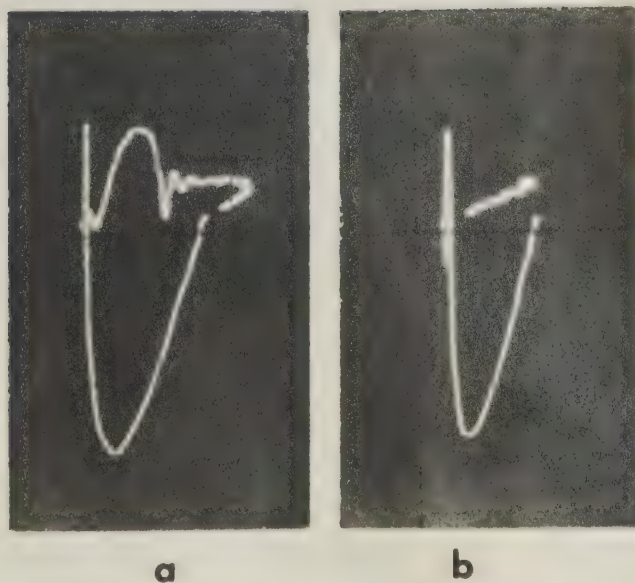


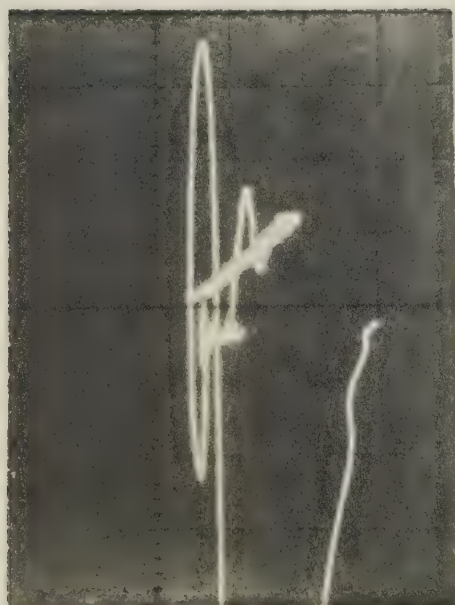
FIGURE 23. Resistance loop before (a) and after (b) subtraction of compliance component. U-shaped portion in (b) is due to mechanical artifact.



a



c



b

- a) Control
- b) After 12.8 μg histamine
- c) Loop 'b' with $> P_{CL}$ subtracted

FIGURE 24. Resistance loop with P_{CL} subtracted.

the resistance loop, with compliance component subtracted, before (a) and after (b) 12.8 μg of histamine were injected. Figure 24c shows the same loop when an amount of pressure greater than that due to the compliance component was subtracted.

In order to eliminate the effects of spontaneous breathing on R_L and C_L , pancuronium bromide was injected at the start of each experiment to paralyze the respiratory muscles of the animal. This drug had no observable effects on either R_L or C_L (see Figure 25). Injections of 0.2, 0.4 or 0.8 ml of saline also produced no significant changes in either R_L or C_L .

In these experiments, only doses of 40 mg/kg diazoxide, 30 mg/kg aminophylline and 0.07 μg INA were tested. Immediately after injection, D caused a moderate fall in compliance and a marked increase in flow resistance. In contrast, neither aminophylline nor INA produced a significant change in either R_L or C_L (see Figure 26).

Injection of histamine produced dose-related decreases in C_L and increases in R_L . Diazoxide effectively inhibited changes in R_L and C_L induced by small doses of histamine. However, if large doses of histamine were injected, only the effects of histamine on C_L were inhibited; no protection from the increases in R_L were noted. In Figure 27, the data from three animals is summarized. All three animals received a low dose of histamine (1.6 μg) before and after diazoxide was administered. The changes in both R_L and C_L were significantly reduced in the presence of diazoxide. Only one animal was given a dose of 12.8 μg of histamine. The change in compliance was reduced after diazoxide had been given but the increase in R_L was not

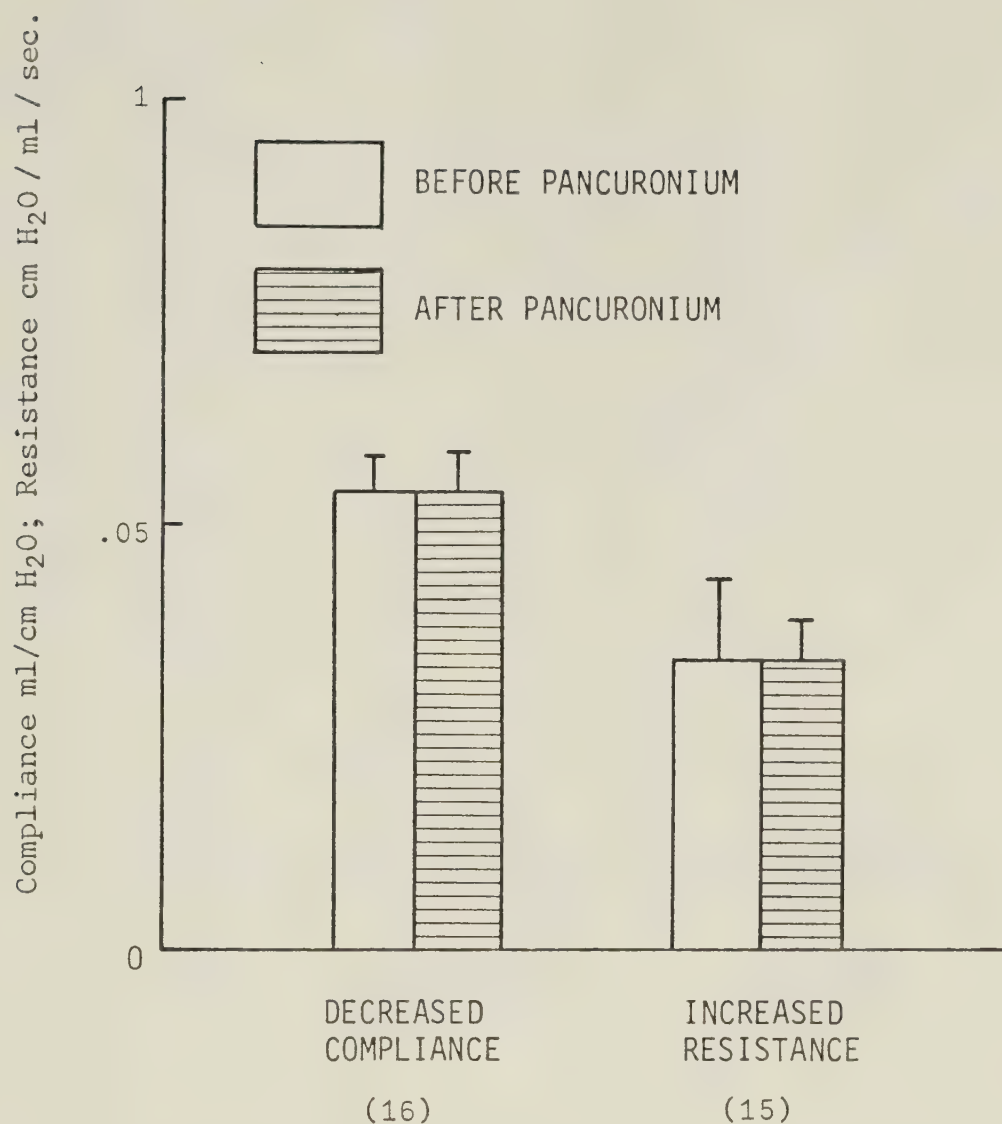


FIGURE 25. Effects of pancuronium on compliance and resistance.

Bars represent mean values before (open) and after (shaded) administration of 0.1 mg/kg pancuronium. Vertical lines show S.E. and n is given in parentheses.

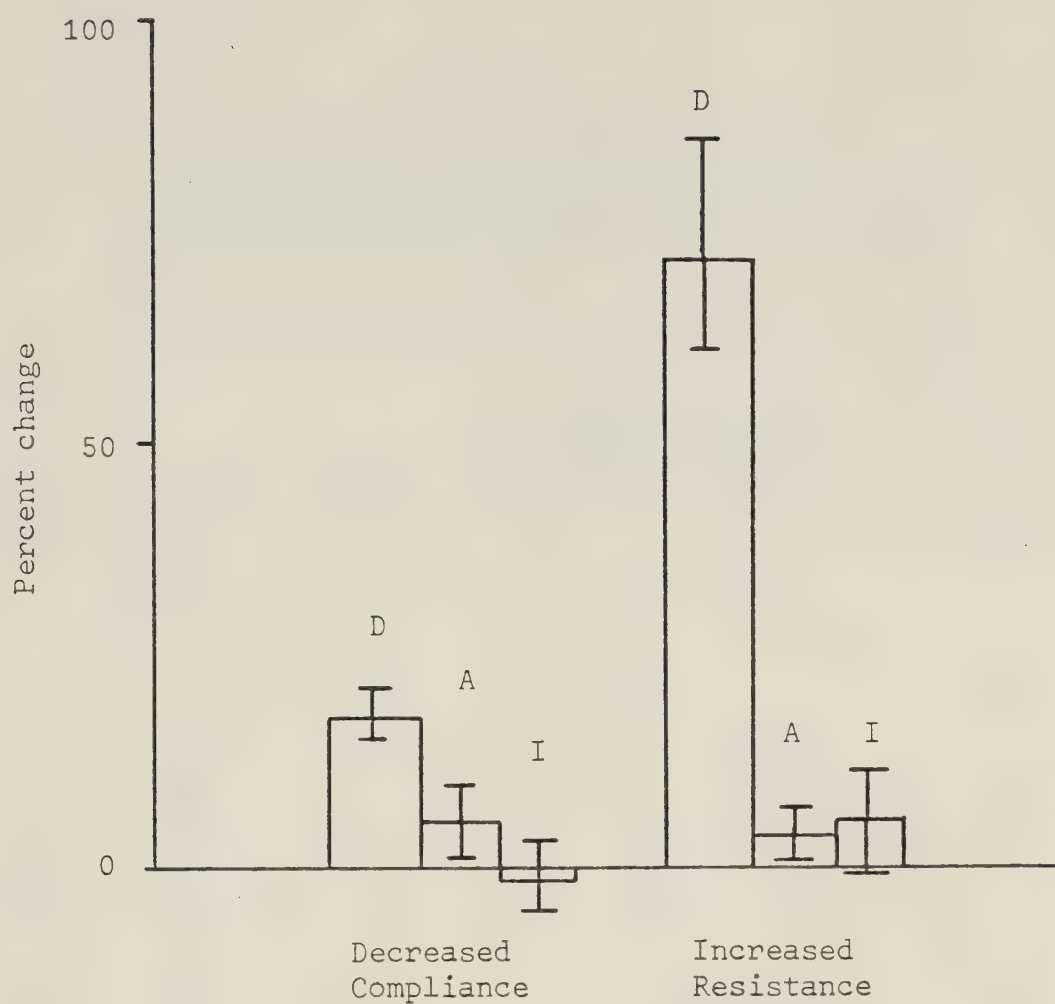


FIGURE 26. Initial effects of diazoxide, aminophylline and INA on compliance and resistance.

Bars show mean of three values after administration of diazoxide 40 mg/kg (D), aminophylline 30 mg/kg (A), or INA 0.07 μ g/kg (I). Vertical lines show S.E.

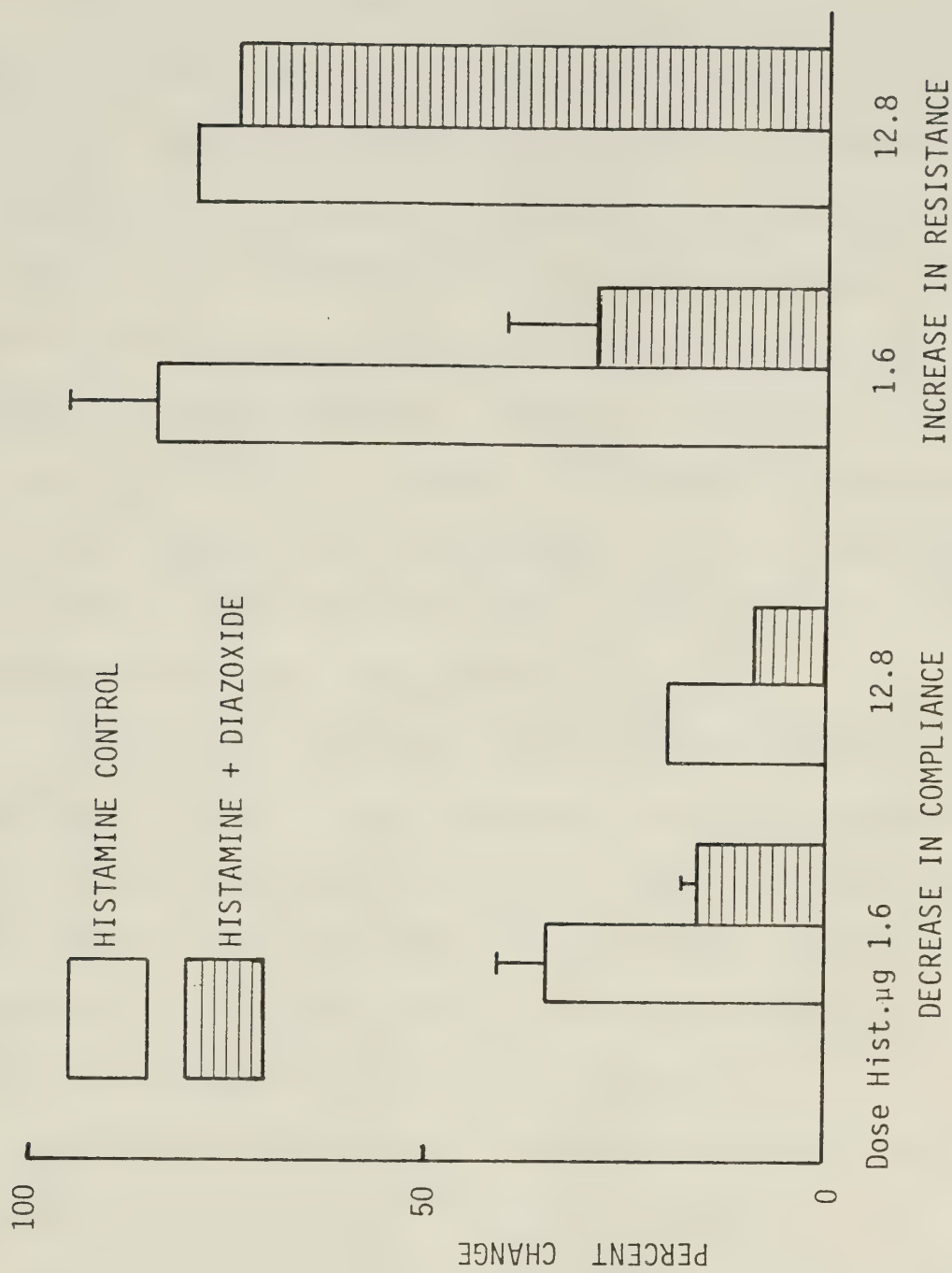


FIGURE 27. Effects of Diazoxide, 40 mg/kg, on histamine-induced changes in compliance and resistance.

The response shown to 1.6 µg histamine represents the mean obtained in 3 animals. Vertical lines show S.E. Only 1 animal received 12.8 µg histamine.

significantly affected. Similar results were obtained with aminophylline and histamine.

Imidazole (I) is a PDE activator (e.g. Butcher & Sutherland, 1962; Kukovetz & Pösch, 1967; Farmer & Farrar, 1976) and would therefore be expected to antagonize actions resulting from the inhibition of PDE. The effects of imidazole on the activity of diazoxide are summarized in Table 5. The interanimal variation among these three animals was so large that no statistically significant differences could be detected between any of the responses. However, in 'animal 2' the effects of diazoxide appeared to be reversed. The response to histamine in this animal was larger after the injection of imidazole than after control injections of histamine. The action of diazoxide in the remaining two animals did not appear to be affected by imidazole.

The effects of 5-HT were also investigated, and these results are summarized in Table 6. The injection of 5-HT caused compliance to fall and flow resistance to increase. The changes in R_L were more marked and occurred after lower doses of 5-HT than the changes in C_L . Diazoxide failed to antagonize the effects of high doses of 5-HT on C_L but did reduce their effects on R_L (Table 6).

Aminophylline prevented the effects of 5-HT on C_L but failed to antagonize the effects of 5-HT on R_L (see Table 7).

The remaining experiments were performed to determine more about the initial changes in R_L and C_L observed after the administration of diazoxide.

Vagotomy or pretreatment with atropine, 0.05 mg/kg, were used to investigate the possible involvement of vagal reflexes or other

TABLE 5

% Decrease in C_L after 6.4 μ g Histamine

Animal	% C_L Decreased			Time (min) Between D & I
	Before D	After D	After D & I	
1	42.4	36.4	33.9	13
	41.4			
2	18.5	12.9	34.5	24
	17.8	21		
3	50.1	37.7	41.2	24
	48.5			
1-3*	36.45	27	36.5	
	(S.E.=5.95)	(S.E.=6)	(S.E.=2.3)	

* Differences not statistically significant

TABLE 6

The Influence of Diazoxide on 5-HT Responses

μg 5-HT	% Change $\pm$ (S.E.)			
	$\downarrow C_L$		$\uparrow R_L$	
	Before D	After D	Before D	After D
2	1.5 $\pm$ (3.2)*	4 $\pm$ (4)	31.9 $\pm$ (3.3)	3.1 $\pm$ (1.6)**
4	12.6 $\pm$ (4.3)	12.5 $\pm$ (1.2)	61.2 $\pm$ (4)	9.1 $\pm$ (4.6)**
8	15.9 $\pm$ (14.5)	25.8 $\pm$ (1.3)	102.7 $\pm$ (20.9)	63.8 $\pm$ (20.9)

* This value represents an increase

** Statistically significant reduction

TABLE 7

The Influence of Aminophylline on 5-HT Responses

μg 5-HT	% Change $\pm$ (S.E.)			
	$\downarrow C_L$		$\uparrow R_L$	
	Before A	After A	Before A	After A
4	10.3 (1)	0.8* (0.8)	31.8 (4.6)	25 (15.9)
8	6.8 (1.1)	6.3	59.1 (4.6)	38.7 (11.4)
16	53.2	7.8*	54.5	54.5

* Statistically significant reduction

parasympathomimetic actions. Neither treatment had any effect on the diazoxide-induced changes in R_L or C_L (see Figure 28). R_L and C_L were not measured before vagotomy, so the effects of vagotomy on these parameters could not be determined. The dose of atropine used in these experiments produced no significant change in either parameter.

The diazoxide-induced changes in R_L were effectively eliminated by pretreatment with mepyramine, 0.1 mg/kg, whereas changes in C_L were unaffected (see Figure 29). This data suggested that histamine was involved in the initial effect on the large airways. The effects of mepyramine on the 5-HT response were also determined in order to check the specificity of mepyramine at this dosage. The responses to 5-HT were unaffected by pretreatment with mepyramine (see Table 8).

Pretreatment with indomethacin, 0.1 mg/kg, produced results opposite to those obtained with mepyramine (see Figure 29). The changes in R_L produced by diazoxide were unaffected but the changes in C_L were abolished. Similar results were obtained in animals pretreated with ASA (see Figure 29). As the initial response to diazoxide resembled the effects of 5-HT, that is, a marked increase in R_L with a less marked fall in C_L , the effects of indomethacin on the response to 5-HT was also investigated. Both the fall in C_L and the increase in R_L produced by 5-HT were potentiated by indomethacin (see Figure 30). These results suggest that 5-HT is not involved in the initial responses to diazoxide, but that a prostaglandin or prostaglandin-like substance is involved with the response to diazoxide which occurs in the small airways.

TABLE 8

Effect of Mepyramine (M)¹ on 5-HT Responses

μg 5-HT	% Change $\pm$ (S.D.)			
	$\uparrow R_L$		$\downarrow C_L$	
	Before M	After M	Before M	After M
2	22.1 $\pm$ (8.2)	17.9		
4	46 $\pm$ (17.8)	42.9		
8	79.6 $\pm$ (15.6)	77.7 $\pm$ (13.2)	35.1 $\pm$ (10.9)	44.6

1. Mepyramine, 0.1 mg/kg.

TABLE 9

Analyses of Blood Samples

Arterial ¹	pH	pCO ₂	pO ₂
Initial (n=3)	7.4 (.06)	33.33 (1.5)	48 (2.65)
Before dilator (n=2)	7.4 (.01)	25.7 (2.55)	53.5 (6.36)
Venous			
Initial	7.49	29.6	28
Before dilator	7.41	31.5	27

1. Measurements $\pm$ S.D.

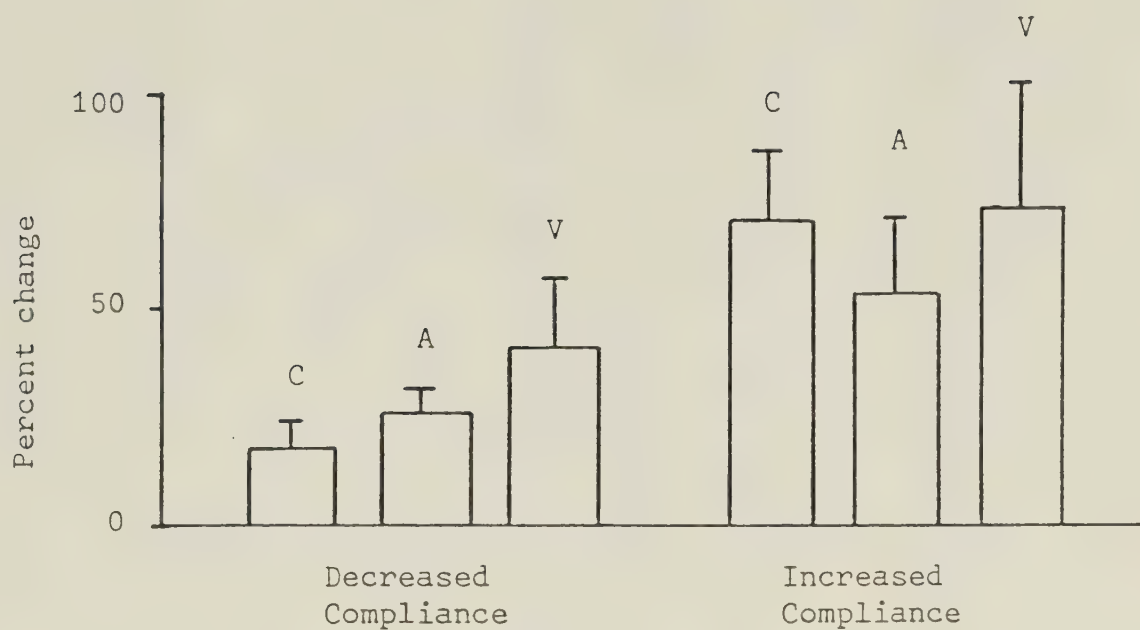


FIGURE 28. Effects of atropine and vagotomy on initial responses to diazoxide.

Bars represent mean percent change produced by diazoxide 40 mg/kg in 3 control animals (C), 4 atropinized animals (A) and 3 vagotomized animals (V). Vertical lines show S.E.

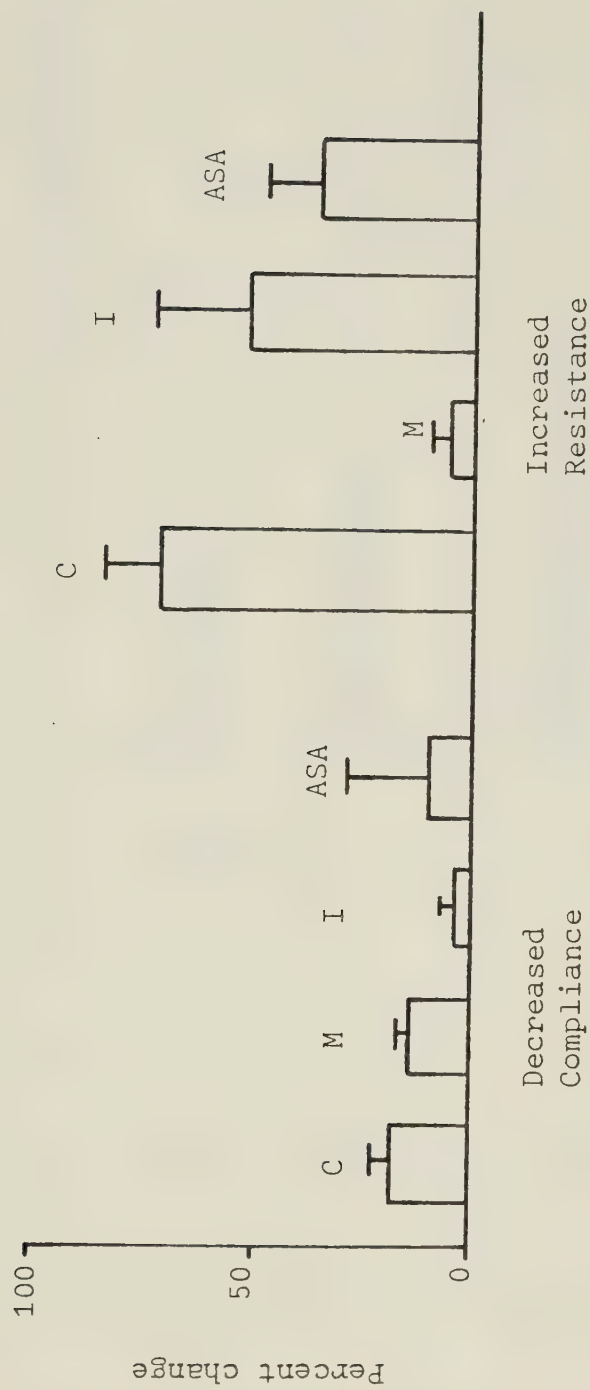


FIGURE 29. Effects of mepyramine, indomethacin and ASA on initial responses to diazoxide.

Bars represent mean % change produced by diazoxide in control animals (C) and those pretreated with mepyramine (M), indomethacin (I) or ASA. Vertical lines show S.E., $n=3$.

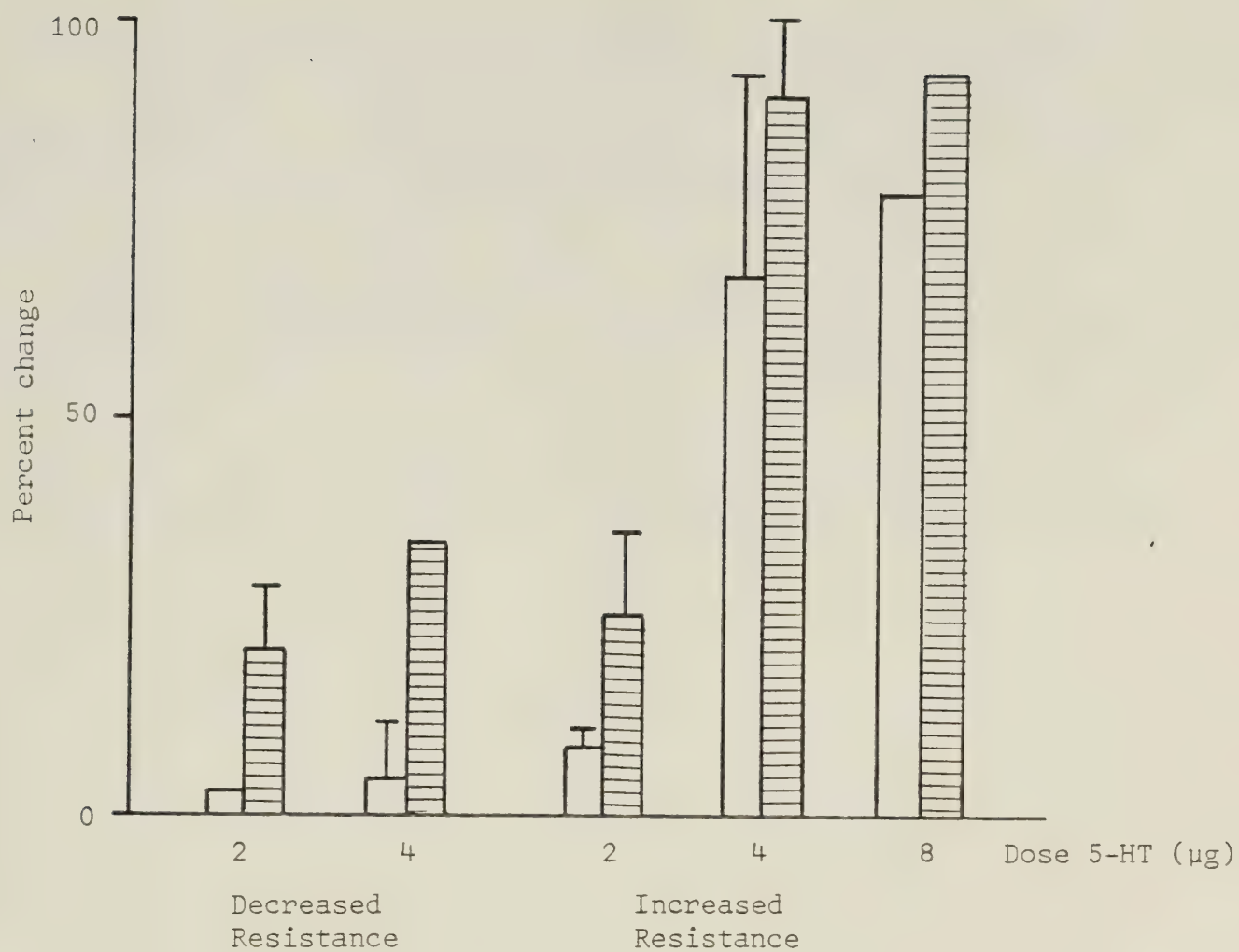


FIGURE 30. Effect of indomethacin on responses to 5-HT.

Bars represent mean change caused by 5-HT before (open) and after (shaded) administration of indomethacin, 0.1 mg/kg.

E. BLOOD SAMPLES

The values of pH and pO_2 obtained from arterial blood samples taken at the start of each experiment were not significantly different from those obtained from samples drawn just before addition of the bronchodilator to be tested. However, a slight degree of hypocapnia was apparent. In the one animal in which venous samples were obtained, the difference between samples was slight. These results are summarized in Table 9. This data suggests that the effects measured were due to the drugs being administered and not to reflexes resulting from changes in blood gases or pH.

DISCUSSION

The relaxation of normal tone in the guinea pig tracheal chain preparation induced by diazoxide or aminophylline was much slower to develop and smaller in magnitude than that produced by INA. Such differences between the response to aminophylline and INA have been reported in the literature (Hawkins & Schild, 1951). As the mechanisms of action of INA and aminophylline are different (e.g. Butcher & Sutherland, 1962; Robison, Butcher & Sutherland, 1967), these results suggest that diazoxide is producing relaxation in a manner similar to aminophylline, that is, by inhibiting PDE, and not in a manner similar to INA.

If the actions of diazoxide were due to inhibition of PDE, one would have expected diazoxide to potentiate the relaxation induced by INA. Neither diazoxide nor aminophylline appeared to potentiate the response to INA in these experiments. However, the maximum response had not been determined and it is possible that no potentiation was detected because the maximum amount of relaxation had been achieved with INA alone. In more carefully controlled experiments, diazoxide markedly potentiated the effects of the β_2 -agonist, salbutamol. Also, in the experiments with histamine-induced tone, the expected potentiation was observed when either diazoxide or aminophylline was combined with INA.

As diazoxide has been reported to cause the release of catecholamines (e.g. Loubatières, Mariani & Alric, 1968; Senft, 1968; Tabachnick & Gulbenkian, 1968), its actions on the tracheal chain were examined in the presence of propranolol. The dose of propranolol employed, 6.8×10^{-7} M, produced an effective blockade of the

β -adrenergic receptors and doses of INA as high as 2×10^{-7} M failed to induce even a slight degree of relaxation. The actions of diazoxide on either normal tone or histamine-induced tone were unaffected by propranolol pretreatment and similar results were obtained with aminophylline. The dilating effect of diazoxide on airway smooth muscle is clearly independent of β -adrenergic stimulation, and these results support the idea that diazoxide is producing relaxation by an action different from that of INA. Although far from conclusive, the results from the tracheal chain preparations would support the hypothesis that diazoxide was relaxing airway smooth muscle by inhibiting phosphodiesterase and thus increasing the level of cAMP.

It is interesting to note that the effects of diazoxide in vivo were not significantly affected by imidazole, a PDE-activator. These results might suggest that diazoxide relaxes airways smooth muscle by a mechanism not involving PDE-inhibition. However, Kukovetz & Pösch (1967) found that the time intervals between administration of the PDE-inhibitor and imidazole were critical to observing the effects of imidazole. In this study only three animals were tested and the optimum time for measuring responses may have been missed.

Too few experiments were done with human tissue to draw any conclusions about the activity of diazoxide in the airway smooth muscle in man. However the variation of responses in these experiments and the length of time required for the tissues to recover illustrate some of the disadvantages of working with isolated preparations. Although the actions and relative potencies of bronchodilators are thought to be similar in guinea pig tracheal and human bronchial tissues (Hawkins &

Schild, 1951; McDougal & West, 1953), the variation observed in these tissues also emphasizes the caution that must be exercised when trying to predict the actions of a drug on human airways from the results of experiments done with animals.

The ability of diazoxide to relax normal and histamine-induced tone in vitro suggested that it might be an effective bronchodilator in vivo. The air overflow experiments were performed to investigate this possibility.

In the air overflow experiments, histamine produced an increase in total lung resistance to inflation (R_{TL}), evidenced by an increased overflow volume, in each animal tested. Although a large interanimal variation was observed, it was much less than that reported in the literature. The air overflow method is unable to distinguish between effects occurring in the small or large airways. However, this technique is thought to be most sensitive to changes in the small airways and, thus, to reflect changes in lung compliance (Holgate & Warner, 1960; Collier & James, 1967; Collier, 1968). This concept is supported by the fact that recovery can be hastened by occlusion of the overflow tube, as lung distension is known to increase compliance (Mead & Whittenberger, 1953; Mead, 1961; Nadel, 1965; Karczewski & Widdicombe, 1969b; Colebatch, 1970; Guyton, 1971; Drazen & Austen, 1974). Therefore, drugs which inhibit the histamine-induced increases in total lung resistance in this type of preparation can be expected to cause dilation of the small airways, although additional actions on the larger airways are not excluded.

Diazoxide was approximately equiactive with aminophylline in preventing histamine-induced increase in R_{TL} in vivo. It is interesting to note that when the order of administration was examined, combinations, in which either diazoxide or aminophylline was given first, were more effective than those in which INA was the first drug administered. The order of administration may also have affected the results obtained when investigating the actions of combinations of relaxants on the tracheal chains.

To this point it appeared that diazoxide might be an effective drug to treat acute bronchoconstriction such as that occurring in an asthmatic attack. However, the ability of diazoxide to produce an initial increase in the air overflow volumes suggested that it possessed a bronchoconstricting action which would limit its usefulness in a patient already suffering some degree of respiratory distress. The measurements of C_L and R_L were particularly helpful in studying this effect as they provided information about which part of the airways was affected.

In order to eliminate the effects of the respiratory muscles, pancuronium bromide was administered at the start of each experiment in which compliance and flow resistance were measured. This muscle relaxant was chosen for several reasons. Firstly, it is a non-depolarizing muscle relaxant and, secondly, it possesses a relatively long duration of action. Only two animals used in these experiments required an additional injection of pancuronium during the course of an experiment. Also, pancuronium is thought to produce little ganglionic blockade and minimal histamine release. In these

experiments, it paralyzed the respiratory muscles without having any direct effect on either R_L or C_L (see Figure 25).

Histamine produced dose-related increases in R_L and decreases in C_L . Although the variation in the response to histamine was greater than that observed in the air overflow experiments, it was still much less than that reported in the literature. The variation could probably have been further reduced by using weight-correlated doses.

Whereas INA prevented histamine-induced changes in C_L and R_L equally, D and A each prevented the histamine-induced decreases in C_L more readily than the increases in R_L . These results suggest that both A and D produce a bronchodilation that primarily occurs in the small airways and becomes less effective as the airway size increases. If this were true, it might also account for the slow and moderate actions of D and A on the tracheal chain preparations. However, the results obtained when 5-HT was the constrictor used must also be considered. These results support the idea that D and A dilate small airways, but suggest that D is also able to relax effectively the smooth muscle in the larger airways. It would appear from these results that the relaxant action of diazoxide on the large airways is dependent on the constrictor used. The inability of diazoxide to relax the large airways when histamine is used as a constricting agent may be related to its initial effects on the large airways. One possible explanation for this will be proposed after the initial effects of diazoxide have been discussed.

Diazoxide produced an initial increase in air overflow volume or total lung resistance to inflation by a combination of effects

on the small and the large airways. Constriction in the small airways appeared to be much less severe than that occurring in the large airways as evidenced by moderate decreases in C_L and very marked increases in R_L .

Neither vagotomy nor pretreatment with atropine produced a significant change in the initial responses to diazoxide. Thus, it is unlikely that these responses involved either vagal reflex activity or the release of acetylcholine.

Pretreatment with mepyramine selectively abolished the increase in R_L observed after diazoxide injection. This suggested that a release of histamine or an histamine-like activity of diazoxide was a factor in producing the increase in R_L but not the decrease in C_L . It would seem that mepyramine was specifically inhibiting an histamine response, as responses to 5-HT were not affected.

In contrast, pretreatment with indomethacin selectively eliminated the increase in C_L observed after diazoxide administration. This suggested that the fall in C_L was mediated by a prostaglandin or prostaglandin-like substance. Similar results obtained in animals pretreated with ASA provide further support to the idea of a prostaglandin-like activity being involved with the initial response to diazoxide in the small airways.

The possible diazoxide-induced release of histamine in the large airways might explain the inability of diazoxide to cause dilation in the large airways when histamine was used as the constricting agent.

A consideration of the cAMP response to histamine provides a possible mechanism for this effect.

Endogenous or exogenous histamine produces an increase in cAMP levels in normal and antigen-sensitized guinea pig lungs, which peaks in approximately 4 min (e.g. Mathé, Sohn & Volicer, 1978). In guinea pig lungs, the increase in the cAMP appears to be mediated by histamine H₂-receptors whereas bronchoconstriction is mediated by H₁-receptors (Mathé, Volicer & Puri, 1974). Histamine has also been reported to cause an H₂-receptor mediated rise in cAMP in other systems (e.g. Lichtenstein & Gillespie, 1973). The H₂-blocking agent, burimamide, abolishes the response (Lichtenstein & Gillespie, 1973; Mathé, Volicer & Puri, 1974) whereas H₁-blocking agents either do not block the response (Lichtenstein & Gillespie, 1973) or only partially block the response (Mathé, Volicer & Puri, 1974).

Mathé, Sohn & Volicer (1978) noted that the histamine-induced increase in cAMP was reduced in the antigen-sensitized lungs. They suggested that the reduction was due to histamine receptor desensitization caused by an increased spontaneous release or "leakage" of histamine in the sensitized lungs. In addition, they showed that the response was equally reduced in lungs from non-sensitized animals which had been pretreated with histamine given intraperitoneally one hour before sacrifice. Mathé, Yen, Sohn & Hedqvist had previously shown that spontaneous release of histamine and prostaglandins was increased in sensitized guinea pig lungs. Prior administration of histamine was also shown to inhibit the increase in cAMP observed after antigen-induced histamine release in leukocytes (Bourne, Melmon & Lichtenstein, 1973).

It would therefore seem reasonable to assume that diazoxide, due to an initial release of histamine or direct action on histamine receptors in the large airways, may cause a desensitization of the H_2 -receptors, and thereby depress the rise in cAMP normally seen after histamine injection. It is also interesting to note that the initial increase in R_L produced by diazoxide lasted approximately 5 min which is in close agreement to the time determined by Mathe', Sohn & Volicer (1978) for the development of H_2 -mediated increases in cAMP.

Although aminophylline was observed to increase overflow volumes in some experiments, it was not observed to alter either R_L or C_L . As R_L determinations could be made quite quickly, it is unlikely that changes in R_L would be undetected. However, measurements of C_L took a longer time to accomplish and it is possible that changes did not persist long enough to be detected. As the major actions of aminophylline appeared to be in the small airways, this would seem to be a reasonable explanation.

These results also cast some doubt on the validity of assuming that changes in air overflow volumes in air overflow experiments primarily reflect changes in small airways. Although inhibition of overflow volumes could be correlated with protection of small airways by changes in C_L , the increased volumes observed after diazoxide injection appear to be affected more by changes in large airways, that is increases in R_L , than by actions on the small airways, that is decreases in C_L .

If it is assumed that changes in R_L result from actions on the trachea and large bronchi, whereas C_L is chiefly affected by actions

at the level of the respiratory bronchioles and alveoli, several conclusions can be drawn from these investigations:

1. Diazoxide is an effective relaxant of respiratory smooth muscle in vitro,
2. Diazoxide is an effective bronchodilator in vivo and it appears to act in both the large and small airways,
3. Upon initial injection, diazoxide may constrict both large and small airways,
4. The initial constriction caused by diazoxide does not involve vagal reflex activity or the release of acetylcholine,
5. The initial constriction of the large airways possibly involves the release of histamine, or a direct action on histamine receptors, whereas,
6. The initial constriction of the small airways is probably mediated by a prostaglandin or a prostaglandin-like substance.

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681-686.

APPENDIX 1

Chemicals, Drugs and Solutions for Part I

A. CHEMICALS AND SUPPLIER

Calcium chloride, Fisher, ACS

Dextrose, Fisher, ACS

Magnesium sulfate, Fisher, ACS

Potassium chloride, Baker, A.R.

Potassium phosphate monobasic, Fisher, ACS

Sodium bicarbonate, Baker, A.R.

Sodium carbonate, Fisher, ACS

Sodium chloride, Fisher, ACS

B. DRUGS AND SUPPLIER (Structures shown in Appendix 3 or text)

Acetyl- β -methylcholine chloride, Aldrich

Aminophylline injection, Glaxo

Acetylsalicylic acid (Aspirin), Mallinckrodt

Atropine sulfate, BDH

Diazoxide injection, Schering

Heparin sodium injection, BDH

Histamine phosphate, Fisher

5-Hydroxytryptamine creatinine sulfate complex, Sigma

Imidazole, Raylo

Indomethacin, Sigma

Isopropylnoradrenaline sulfate, Fluka

Mepyramine maleate, Poulenc

Pancuronium bromide injection, Organon

Propranolol hydrochloride injection, Ayerst

Salbutamol sulfate, Allen & Hanburys

Sodium pentobarbital injection, Abbott

Xylocaine hydrochloride injection, Astra

C. KREBS SOLUTION

Krebs solution (Edinburgh Staff, 1968) of the following composition (g/1): NaCl, 6.9; KCl, 0.35; CaCl₂.2H₂O, 0.37; KH₂PO₄, 0.16; MgSO₄, 0.29; NaHCO₃, 2.1; Dextrose, 2, was prepared by dissolving the sodium bicarbonate and then adding the remaining salts except calcium chloride. Calcium chloride was added last to avoid precipitation of calcium carbonate.

D. DRUG SOLUTIONS

1. DIAZOXIDE

For in vitro experiments, 0.2 ml of diazoxide injection, Hyperstat^R, was diluted to a final volume of 10 ml with Krebs solution. The diazoxide concentration of this solution was 3 mg/ml; attempts to prepare stronger solutions resulted in precipitation. Solutions of 3 mg/ml strength would also precipitate after approximately 45 minutes. Therefore, solutions were used within 30 minutes of preparation or discarded. Hyperstat^R was used undiluted for in vivo experiments.

2. STOCK SOLUTIONS

a. Indomethacin

Indomethacin was dissolved in an equimolar solution of sodium carbonate in distilled water to give a final concentration of indomethacin equal to 2 mg/ml. This stock solution was diluted with equal parts of normal saline before use.

b. Pancuronium Bromide

A stock solution of pancuronium bromide, 0.2 mg/ml, was prepared by a 1:10 dilution of pancuronium bromide injection, Pavulon ^R, with normal saline. The solution was not further diluted before use.

c. Mepyramine Maleate

A 1×10^{-3} M solution of mepyramine in distilled water was prepared. Prior to injection, the solution was diluted 1:3 with normal saline.

d. Saline

i. Normal Saline

Normal saline was prepared by dissolving sodium chloride in distilled water to a final concentration of 0.9%.

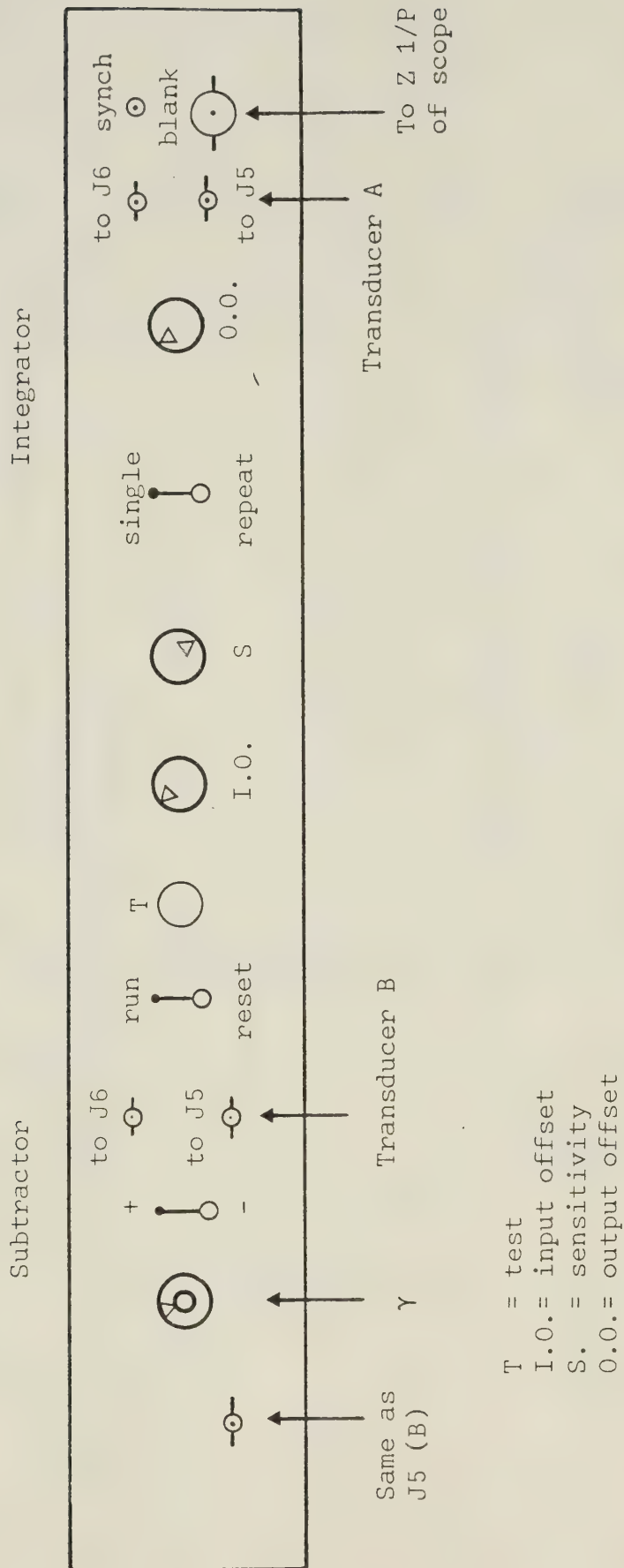
ii. Heparinized Saline

Heparinized saline was prepared by adding sodium heparin injection to normal saline to produce a final concentration of 100 I.U. heparin/ml.

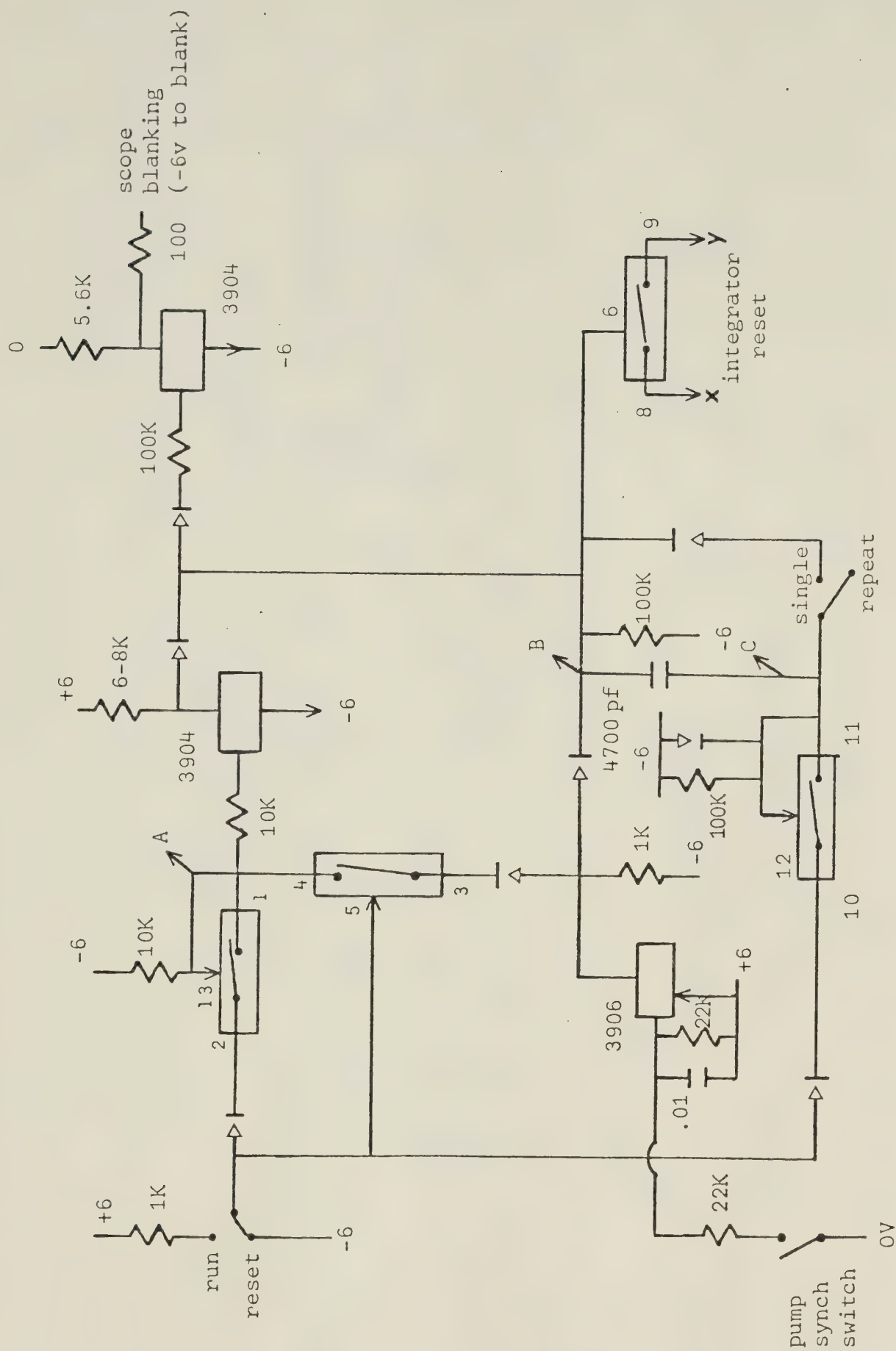
3. MISCELLANEOUS

The remaining drugs used in these experiments were dissolved in Krebs solution or normal saline at the beginning of each experiment. Because of its instability, isopropylnoradrenaline was prepared just prior to each dose given.

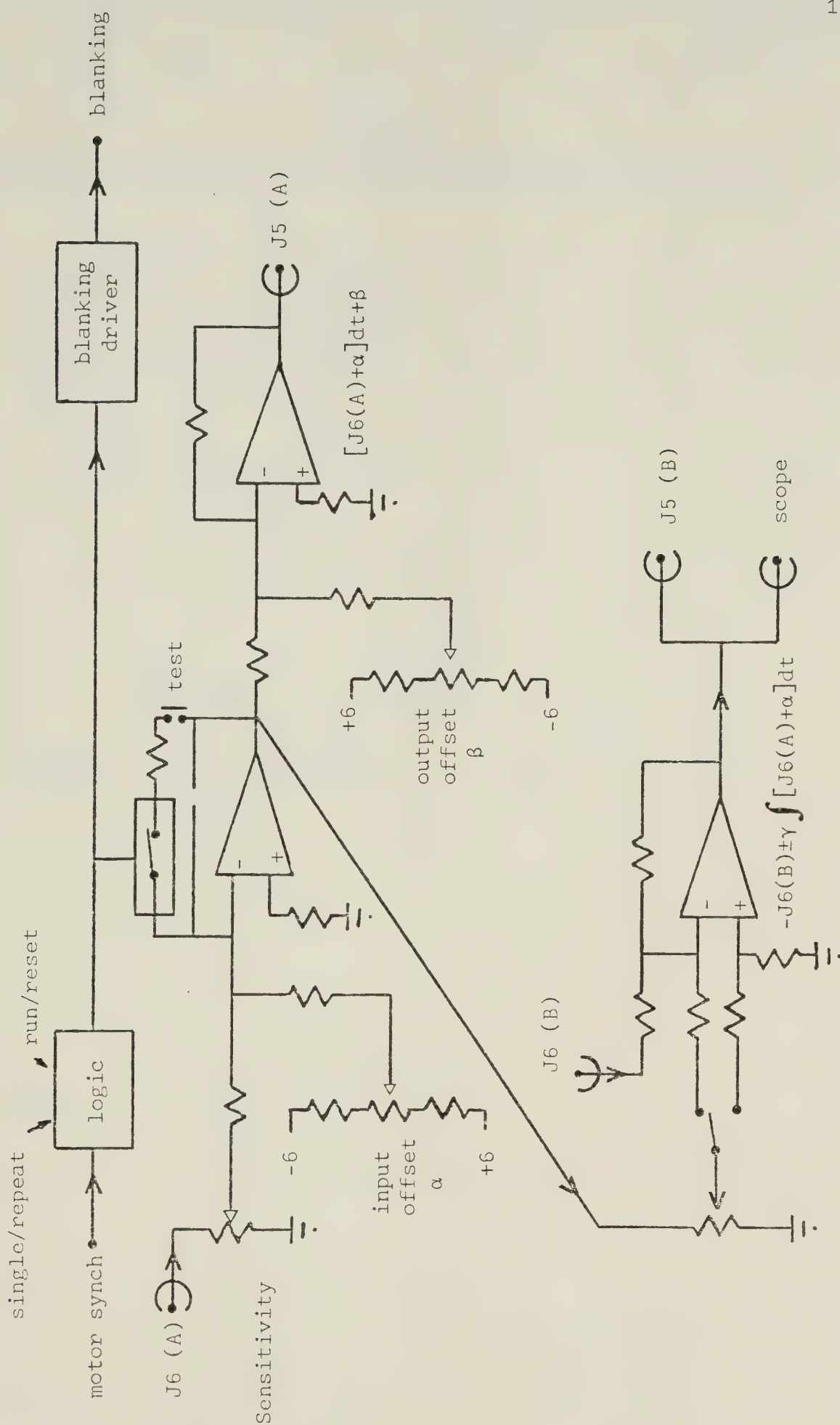
APPENDIX 2



Front panel of integrator/subtractor unit



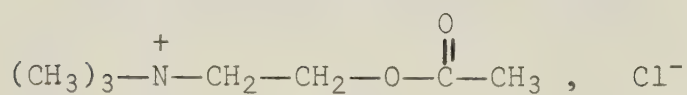
Integrator connections to scope and pump



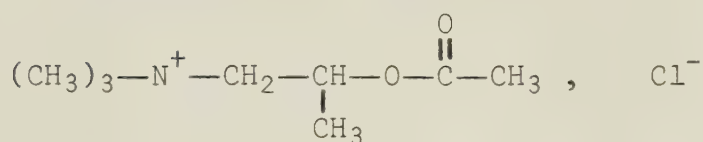
Subtractor/integrator circuit diagram

APPENDIX 3

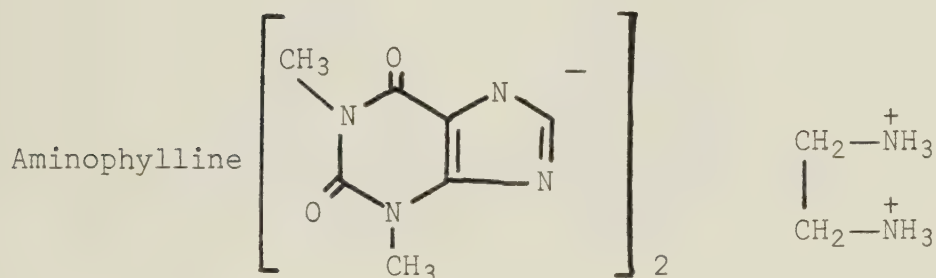
Structures of drugs used in experiments and not shown in text



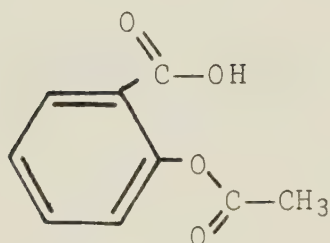
Acetylcholine chloride



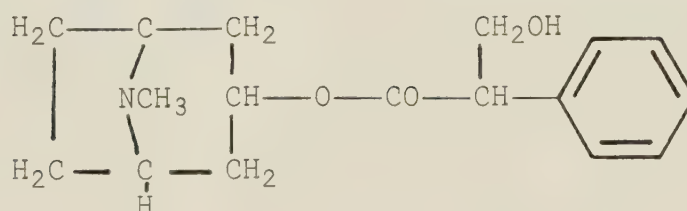
Acetyl-β-methylcholine chloride

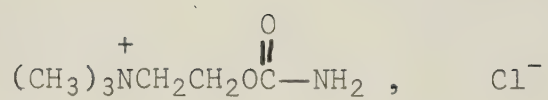


Acetylsalicylic Acid

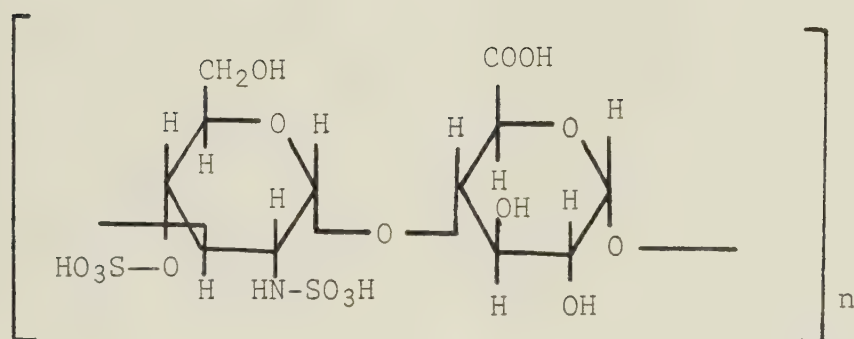


Atropine



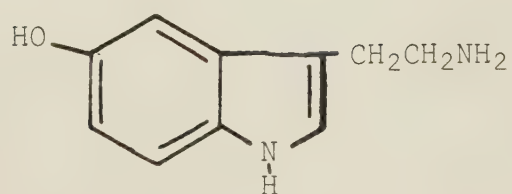
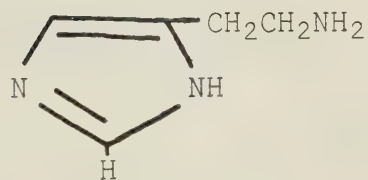


Choline Chloride Carbamate (Carbachol)



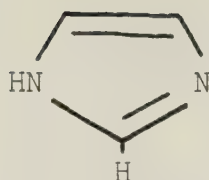
Heparin

Histamine

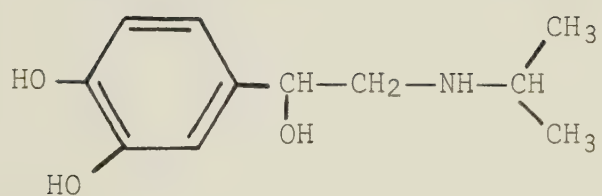
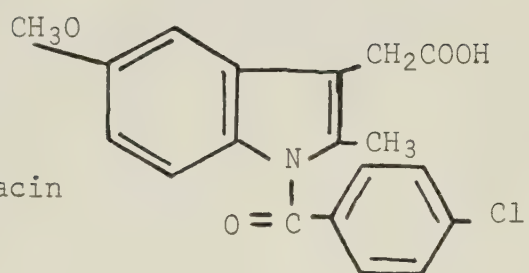


5-Hydroxytryptamine

Imidazole

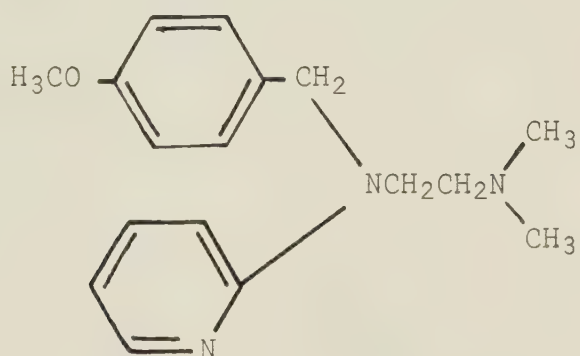


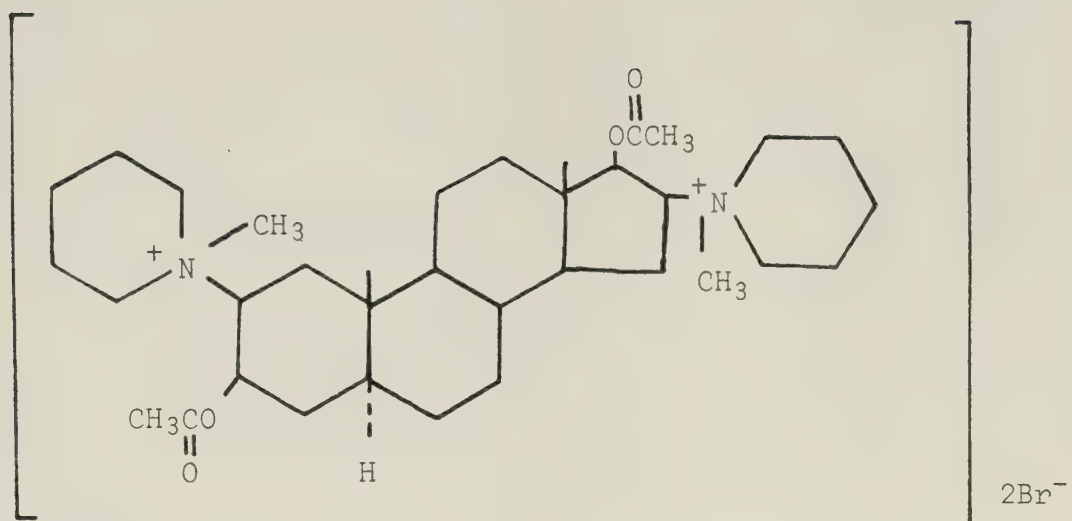
Indomethacin



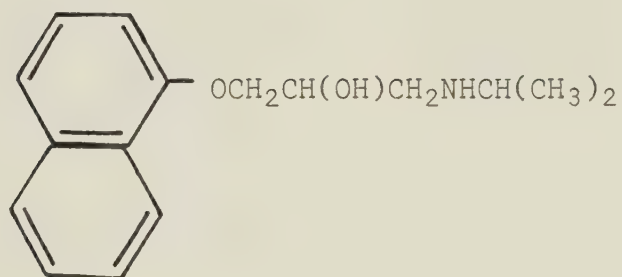
Isopropylnoradrenaline

Mepyramine

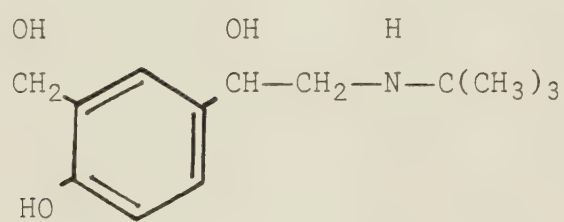




Pancuronium bromide

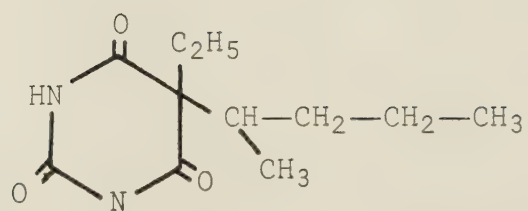


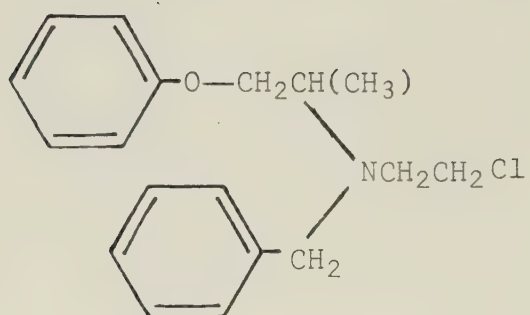
Propranolol



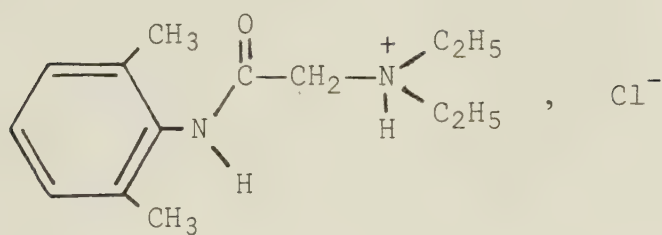
Salbutamol

Pentobarbital





Phenoxybenzamine



Xylocaine hydrochloride

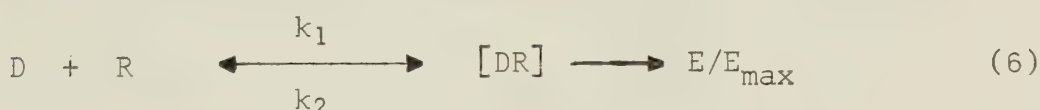
PART II

THE EFFECTS OF HEXAMETHONIUM AT THE MUSCARINIC RECEPTOR
OF ACETYLCHOLINE

INTRODUCTION

This section of this thesis is concerned with the influence of hexamethonium (C_6) on the binding of agonists to the muscarinic receptor of acetylcholine (ACh). In this introduction, various theories of drug-receptor interactions and methods of calculating the binding of drugs to their receptors will be discussed. Interactions at the muscarinic receptor for acetylcholine will be stressed and a rationale for these investigations provided.

Structurally specific drugs are believed to exert their pharmacological effects only after combining with a particular cellular component (e.g. Goldstein, Aronow & Kalman, 1969; Korolkovas, 1970). The cellular component which combines with the drug is referred to as the drug receptor. The structure of the drug must be highly complementary to that of the receptor before binding will occur and a drug-receptor complex is formed. The formation of the complex is governed by the law of mass action (Clark, 1937), and can be described by the following reversible equation:



where D is the drug; R is the receptor; DR is the drug-receptor complex; k_1 and k_2 are the forward and reverse rate constants, respectively; E is the pharmacological effect of the drug, and $E_{\max}$ is the maximal effect possible.

The ratio of k_1/k_2 is defined as the affinity constant (K_{AFF}) of the drug, and its reciprocal is defined as the dissociation constant (K_D) of the drug. Thus, either K_{AFF} or K_D can be used as an index of

the binding of a drug to its receptor. It should be noted that more than one step may be required between the formation of the drug-receptor complex, DR, and the occurrence of the pharmacological effect of the drug. A variety of theories have been proposed to try and define the relationship between the formation of the DR and the production of the pharmacological response (e.g. Furchgott, 1964; Van Den Brink, 1977). The Occupation Theory, Rate Theory, Rang's two-state model, and a multi-state model will be briefly reviewed in this introduction.

A. OCCUPATION THEORY

The Occupation Theory of drug action was based on the assumption that the magnitude of the pharmacological effect was proportional to the fraction of receptors occupied by the drug, with the maximal response occurring when all the receptors were occupied (Clark, 1937). As the interaction of drug and receptor follows the law of mass action, the binding of the drug to the receptor is defined by the term:

$$k_1 [D] (1-y)$$

where k_1 is the forward rate constant; $[D]$ is the concentration of the drug; and y is the fraction of receptors already bound to the drug. Similarly, the dissociation of the drug-receptor complex is defined by the term:

$$k_2 y$$

where k_2 is the reverse rate constant; and y again refers to the fraction of receptors bound to the drug.

At equilibrium, the rate at which the drug binds to the receptor is equal to the rate at which it leaves the receptor and, thus,

$$k_1[D] (1-y) = k_2[D] \quad (7)$$

Rearrangement of equation 7 results in the following equation:

$$k_2/k_1 = [D](1-y)/y = K_D \quad (8)$$

When 50% of the receptors are occupied, $y = 0.5$ and equation 8 simplifies to $K_D = [D]$. As the response is assumed to be proportional to the fraction of receptors occupied, $E/E_{\max}$ will also equal 0.5 and K_D can therefore be measured as the concentration of drug which produces 1/2 of $E_{\max}$. Thus, the smaller the K_D (or the larger the K_{AFF}), the smaller the amount of drug required to produce a response, and the more active is the agonist.

The only requirement for drug action, according to this theory, is that the receptor must be occupied by the drug. The existence of partial agonists, antagonists, the phenomenon of desensitization or fade could not be explained. The introduction of the concepts of intrinsic activity (Ariens, 1954) and efficacy (Stephenson, 1956) developed the Occupancy Theory so that it provided an explanation for the fact that various drug-receptor complexes possessed unequal abilities to initiate pharmacological effects. Antagonists could then be described as drugs which possessed affinity for the receptor but, once bound, were unable to initiate a response. Conversely, not all agonists would have to occupy all of the receptors to produce a maximal response. In addition, because $E_{\max}/2$ could presumably be obtained by some agonists without

one-half of the receptors being occupied; the value of K_D was no longer equal to the concentration of drug producing the half-maximal response. A method of calculating K_D , based on the idea of efficacy, was reported by Furchgott (1966) and will be discussed later.

However, even with the introduction of these concepts, the Occupancy Theory was still limited by its inability to explain why one drug was an antagonist while another drug, closely related in structure to the first, was an antagonist or partial agonist. The phenomenon of desensitization also remained unexplained. Both of these limitations were overcome to some extent by the Rate Theory proposed by Paton (1961).

B. RATE THEORY

According to the Rate Theory of drug action, the pharmacological effect is initiated by the combination of drug and receptor rather than by the drug-receptor complex (Paton, 1961). The drug-receptor interaction is again assumed to follow the law of mass action and, therefore, the activity of a given drug is determined primarily by its ability to dissociate from the receptor. A potent agonist can thus be described as a drug that dissociates quickly, thus facilitating many interactions in a given time, whereas a weak agonist or antagonist dissociates much more slowly. According to this theory, fade is a natural consequence of the occupation of some receptors by the drug. The major limitation of this theory was its lack of experimental support in any biological system other than the ileum (e.g. Waud, 1968). Also, Roberts and Stephenson (1976), using guinea-pig ileum, reported that the actions of some antagonists did not conform to the predictions of Rate Theory.

Another model which also provides an explanation for antagonism and desensitization is the Metaphilic Theory or Two-State Model described by Rang & Ritter (1970).

C. TWO-STATE MODEL

Rang & Ritter (1970) suggested that the drug receptor was able to exist in two conformations. One conformer, denoted as R, is the form which combines with the drug. The second conformer, denoted as R', is a desensitized form of the receptor. Once the drug-receptor complex has been formed, some of the receptors may undergo a conformational change to the desensitized form (see Figure 31). Interconversions between R and R' are assumed to occur much more slowly than either the association or the dissociation of drug and receptor. Thus, the magnitude of pharmacological response, the amount of desensitization, or the amount of antagonism can be explained by the relative affinities of the drug to R and R'.

Rang (1973) also discussed a two-state model in which the receptor existed in either its normal 'resting' state or in an 'activated' conformation. Again, the action of a drug depended on its relative affinities for the two forms and how it shifted the original equilibrium between them. Other cyclic models, in which the receptor can exist in more than two conformations, have also been proposed (e.g. Gage, 1976).

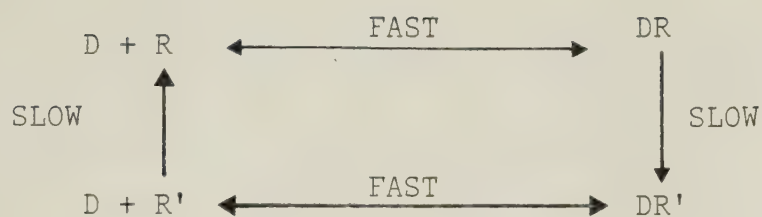


FIGURE 31. Two-state model.

D = drug

R = receptor in normal form

R' = receptor in desensitized form

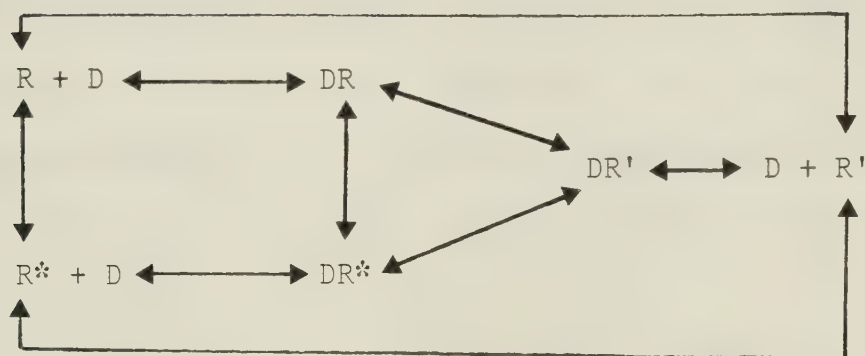


FIGURE 32. Multi-state model.

D = drug

R = receptor in normal state

R' = desensitized receptor

R* = receptor in high affinity state

D. MULTI-STATE MODEL

A multi-state model which incorporates both of the two-state models mentioned above is depicted in Figure 32.

In this model, the receptor can exist in three conformations, denoted as R, R', and R*, which have different affinities for the drug, D. R represents the normal receptor, R' represents a desensitized conformer with little affinity for D, and R* represents an activated receptor with high affinity for D. As in the two-state model, the activity of the drug depends on its relative binding affinities to each of the conformers.

In general, two major approaches have been used to determine the binding affinity (K_D or K_{AFF}) of a full agonist to its receptor:

1. From a comparison of dose-response curves obtained before and after irreversible blockade of a portion of the total receptors (Furchgott, 1966; Furchgott & Bursztyn, 1967), and
2. From measurements of the binding or displacement of labelled drugs, usually using tissue homogenates (Gill & Rang, 1966; see Review, Birdsall & Hulme, 1976).

The two methods of calculating K_D used in these investigations (Furchgott, 1966; Parker & Waud, 1971) are of the first type and will be discussed in detail. Both methods assume that the law of mass action applies to the interaction between drug and receptor, that equal biological responses correspond to equal biological stimuli or receptor activity, and that the concentration of agonist producing a particular response is the same as the concentration of agonist in the

bathing solution, that is, diffusional barriers or access limitations are assumed to be minimal or non-existent.

E. METHOD DESCRIBED BY FURCHGOTT

According to this method, the relationship between the pharmacological effect and the agonist-receptor complex is described by the following:

$$E_A/E_m = f(s) = f(\epsilon[RA]) = f(\epsilon R_T \cdot [RA]/[R_T]) = f(e[RA]/[R_T]) \quad (9)$$

A definition of terms is provided in Table 10.

From the law of mass action, $[RA]/[R_T]$ is defined as $[A]/(K_D + [A])$.

Therefore,

$$E_A/E_m = f(S) = f(\epsilon[R_T] \cdot [A]/(K_D + [A])) = f(e \cdot [A]/(K_D + [A])) \quad (10)$$

Similarly, after irreversible inactivation of a portion $(1-q)$ of R_T , the relationship between the effect and the agonist-receptor complex can be described by:

$$E'_A/E'_m = f(S') = f(\epsilon(q[R_T] \cdot [A']/(K_D + [A']))) = f(qe \cdot [A']/(K_D + [A']))) \quad (11)$$

For equal responses obtained before and after the irreversible blockade, equation 2 can be set equal to equation 3 and one can obtain:

$$1/(A) = 1/q(A') + (1-q)/qK_D \quad (12)$$

The plot of $1/(A)$ against $1(A')$ should therefore produce a straight line with a slope of $1/q$ and an intercept equal to $(1-q)/qK_D$. The value of

TABLE 10

Definition of Terms Used by Furchgott (1966)

E_A	- pharmacological effect or measured response to agonist, A
E_m	- maximal response or effect possible
S	- biological stimulus, described by Stephenson (1956), arbitrarily defined as 1 when $E_A/E_m = 0.5$.
ϵ	- intrinsic efficacy (dimensions of $1/(R_T)$). $S = \epsilon(RA)$
e	- efficacy described by Stephenson (1956). $e = \epsilon(R_T)$.
(R_T)	- initial concentration of total active receptors for A
(R)	- concentration of unbound receptors
(RA)	- concentration of bound receptors or agonist-receptor complex
$(RA)/(R_T)$	- fraction of active receptors bound to agonist
(A)	- concentration of unbound A in region of receptors (assumed to be concentration in bath)
K_D	- dissociation constant of RA with dimensions of A
q	- fraction of (R_T) remaining in active form after irreversible inactivation of $1-q$ receptors
$E_A', E_m', S',$ and (A')	- equivalent to E_A, E_m, S and (A) , respectively, following reduction of (R_T) to $q(R_T)$

K_D can be calculated as (slope-1)/intercept, and the fraction of receptors still active (q) can be calculated as $1/\text{slope}$.

In the method described by Parker & Waud (1971), the double reciprocal plot of $1/(A)$ against $1/(A')$ is only used to provide an estimate of K_D . The estimate obtained is then used to calculate the value of K_D from the non-transformed data.

F. METHOD DESCRIBED BY PARKER & WAUD

The method described by Parker & Waud (1971) is a computerized method and therefore does not require that the data be transformed to a linear form in order to conveniently determine binding parameters. Data from a dose-response curve obtained before occlusion of some of the receptors is fitted to a logistic function using an iterative least squares technique to produce a control curve. Next, responses obtained after a fraction of the receptors have been inactivated are matched to corresponding points on the control curve. The corresponding values of $1/[A]$ and $1/[A']$ are then fit to a straight line, and K_D is estimated as the slope/intercept. The estimated value is then used to begin the iterative fit of A and A' to a hyperbolic function of the form,

$$A = M \frac{A'}{A' + K} .$$

The final hyperbolic parameters of M and K are used to calculate the K_D and q .

As receptors are inactivated, a progressive shift of the dose-response curve to the right is observed before any depression of the

maximal response. According to the methods of Furchgott (1966) and Parker & Waud (1971), this shift indicates a reduction of a receptor reserve. Values of q can be used to determine the fraction of total receptors required to produce a maximal response as the magnitude of the shift of the dose-response curve is assumed to be related to the number of inactivated receptors.

Kenakin & Cook (1976) questioned the existence of spare histamine receptors and offered two alternative explanations for the progressive shift of dose-response curves and the subsequent reduction of the maximal response. They suggested that alkylation of a regulatory site of the receptor could produce a conformational change resulting in decreased affinity for the agonist and therefore a shift of the dose-response curve, whereas alkylation at a separate site produced a reduction in the maximal response. This type of mechanism would be compatible with the two-state or multi-state models of drug-receptor interactions. They also suggested that a quasi-competitive antagonism which could arise from alkylation of a site removed from the receptor, could explain such results.

Earlier, Moran & Triggle (1970) had questioned the existence of spare receptors in adrenergic and cholinergic systems. They obtained data which suggested that the muscarinic receptor of acetylcholine possessed at least two sites, and concluded that allosteric mechanisms, rather than spare receptors, were responsible for the shift in dose-response curves.

Other workers using smooth muscle preparations also reported that the binding of agonists to the muscarinic receptor did not appear to

follow the law of mass action for a single receptor or binding site (Burgen & Spero, 1968, 1970; Sastry & Cheng, 1972; Young, 1974; Haigh & Young, 1975; Taylor, Cuthbert & Young, 1975). Although diffusional barriers may account for some of the discrepancy (Rang, 1966; Thron & Waud, 1968; Roberts & Stephenson, 1974), such results also suggest either a heterogeneous receptor population or a population of receptors containing more than one binding site (Burgen & Spero, 1968, 1970; Sastry & Cheng, 1972; Young, 1974; Haigh & Young, 1975). In contrast, the binding of antagonists at the muscarinic receptor for acetylcholine has been reported to conform to the law of mass action (Birdsall & Hulme, 1976). As the antagonists would be subject to the same access limitations as agonists, these data suggest that barriers to diffusion were not an important factor in the deviations from the law of mass action noted for the binding of agonists. Results obtained from investigation of the binding parameters of agonists using labelled compounds (approach 2) also suggest more than one binding component at the muscarinic receptor of acetylcholine (Burgen & Hiley, 1974; Birdsall & Hulme, 1976; Aronstam, Hoss & Abood, 1977; Chang & Bock, 1977; Kloog & Sokolovsky, 1977). All of these results appear to be compatible with the recent conclusions of Gupta, Moran & Triggle (1976) who suggest that the binding of agonists at the muscarinic receptor involves both a recognition site and an allosteric site. Binding of an antagonist to the recognition site eliminates receptors available for agonists and therefore causes a depression of the maximal response. In contrast, binding at the allosteric site produces conformational changes and, therefore, results in a changed affinity for

agonists. Because the affinity for the agonist is changed, a different amount of agonist is required to produce the same response, and a shift in the dose-response curve can be observed.

Furchgott (1978) reviewed the precautions which must be exercised when determining binding parameters of drugs which are capable of interacting with more than one distinct type of receptor. Sastry & Cheng (1972) had earlier emphasized the necessity of not only blocking the extraneous receptors, but of establishing that the agent used for such a blockade had no effect on the affinity of an agonist to the receptors being examined. It is generally accepted that acetylcholine binds to two distinct groups of receptors: muscarinic and nicotinic. Certain agonists (e.g. acetylcholine or carbachol) may act on both nicotinic and muscarinic receptors. For example, Sastry & Cheng (1972) reported equal values for the K_D of acetylcholine for muscarinic and nicotinic receptors. In contrast, cholinergic antagonists appear to be selective for either the muscarinic or the nicotinic type of receptor. For example, hexamethonium (C_6), a bis-quaternary ammonium compound, possesses (nicotinic) ganglionic blocking activity, whereas atropine selectively inhibits muscarinic receptors.

Acetylcholine also binds to the enzyme acetylcholinesterase (AChE) which shows many similarities to muscarinic receptors (Belleau, 1964; Beckett, 1967; Ehrenpreis, 1967; Wurzel, 1967; Belleau, 1970; Podleski & Changeux, 1970). AChE is a membrane-bound enzyme which has been reported to exhibit actions of a "typical allosteric" enzyme (Changeux, 1966; Belleau, 1970; Kitz & Braswell, 1970; Pavlic, 1975). The activity of AChE has been observed to be affected by both monoquaternary

(muscarinic) agonists and bis-quaternary (nicotinic) agents (Belleau, 1970). In particular, C₆ has been reported to increase the rate at which AChE hydrolyzes acetylcholine (Kensler & Elsner, 1951; Roufogalis & Thomas, 1969). A high degree of structural specificity was required to produce a potentiation of AChE activity, and it was concluded that C₆ produced its effects through an allosteric mechanism at a site peripheral to the catalytic site of the enzyme (Roufogalis & Thomas, 1968; 1969; Belleau, 1970; Roufogalis & Wickson, 1975).

Hexamethonium is often added to the bathing solution during the determination of the binding parameters of various agonists and antagonists to the muscarinic receptor of acetylcholine (Abramson, Barlow, Mustafa & Stephenson, 1969; Sastry & Cheng, 1972; Abramson, Barlow, Franks & Pearson, 1974; Barlow, 1973; Barlow, 1975; Barlow & Casy, 1975; Barlow, Scott & Stephenson, 1963; Barlow, Scott & Stephenson, 1967; Mackay, 1966; Parker, 1972; Roberts & Stephenson, 1976). However, there are few reports of the influence of C₆ on such parameters and those that do exist are inconclusive (Barlow, Franks & Pearson, 1972; Sastry & Cheng, 1972; Barlow, Berry, Glenton, Nikolaou & Soh, 1976). Because of this, and in view of its effects on AChE, this study was undertaken to determine the effect of hexamethonium on the binding of various agonists to the muscarinic receptor for acetylcholine. The possible influence of DMPP, trimethaphan, nicotine, and mecamlamine was also investigated in order to determine the specificity of any effects that might be observed.

MATERIALS AND METHODS

These investigations consist of two series of experiments which will be discussed separately. However, the isolation and preparation of tissues was the same for both series, so will be described first.

Random bred, male guinea pigs weighing between 250-1000 g were starved overnight. An animal was stunned by placing it in an atmosphere of carbon dioxide which had been prepared by placing dry ice in the bottom of a dessicator. Once stunned, the carotid arteries were severed and the animal bled to death. The small intestine was excised from a point approximately 10 cm from the ileocecal junction. Strips of longitudinal muscle were obtained from 10-20 cm lengths of whole ileum by a method similar to that described by Paton & Zar (1968). The longitudinal muscle layer was separated from the underlying tissue by gently scraping a piece of moistened cotton or Q-tip over the entire surface of the piece of ileum and then lifting it free. Although this method yields a relatively non-frayed tissue, there is a stronger possibility of including some of the underlying circular muscle. The muscle strips were then cut and folded in half to form a doubled preparation ranging in length from 1.4 to 3.2 cm (2.2 ± 0.06 S.E., $n=44$) in order to increase the force of contraction. Two preparations were tied to a glass tissue holder and placed in a 7 ml tissue bath containing Krebs solution (Part I, Chapter 2, Appendix 1-c) gassed with 5% CO₂ / 95% O₂ and maintained at 37°C. The free end of the tissue was attached to a Grass Force Displacement Transducer Model FT03C connected to a Grass Model 7D Polygraph. An initial resting tension of 500 mg was applied to each preparation.

The tissues were allowed to equilibrate for one hour and then drugs were injected into the bath according to the two minute cycle shown in Figure 33. Tissues were washed according to the cycle during equilibration periods. Washes were automated and consisted of 30 ml of prewarmed Krebs solution pumped from the bottom of the tissue bath.

Drugs and chemicals used in these experiments are listed in Appendix 4. All drugs used in these experiments were dissolved in Krebs solution. Those injected into the tissue baths were added in volumes ≤ 0.4 ml.

A. SERIES A EXPERIMENTS

In this series of experiments the K_D 's of acetylcholine and carbachol were determined using unpaired preparations. Tissues bathed in a normal Krebs solution served as controls. Test preparations were those in which the Krebs solution contained one of the ganglionic blocking agents listed in Table 11.

Nicotine, trimethaphan, DMPP, and mecamylamine represent different chemical classes and were included to determine if any differences observed were specific to hexamethonium (see Figure 34).

After the tissues had equilibrated for one hour, a single dose of either acetylcholine or carbachol was repeated until uniform responses were obtained. Dose-response curves to both agonists were then obtained with three 'rest-cycles' allowed before and after each new agonist was tested. During the 'rest-cycles' no drug was injected but the tissues were washed as usual. After the last rest-cycle, the tissues were

FIGURE 33
Cycle Used for Drug Injections

Arcs	Seconds	Event
0	0	Drug injected
0 - 50	0 - 16.7	Drug in contact with tissues
50 - 90	16.7 - 30	Wash
90 - 120	30 - 40	Rest
120 - 160	40 - 53.3	Wash
160 - 360 $\equiv$ 0	53.3 - 120 $\equiv$ 0	Rest

TABLE 11

Ganglionic Blocking Agents Used in Series A Experiments

Agent	Concentration
Hexamethonium chloride*	1×10^{-4} M
" *	2×10^{-4} M
" *	4×10^{-4} M
Nicotine dihydrochloride	3.2×10^{-4} M
Trimethaphan camphorsulfonate*	3.4×10^{-5} M
" *	1.7×10^{-5} M
" *	8.4×10^{-6} M
DMPP *	6.3×10^{-5} M
Mecamylamine hydrochloride	9.8×10^{-5} M
"	4.9×10^{-5} M
"	2.5×10^{-5} M

* Also used in Series B.

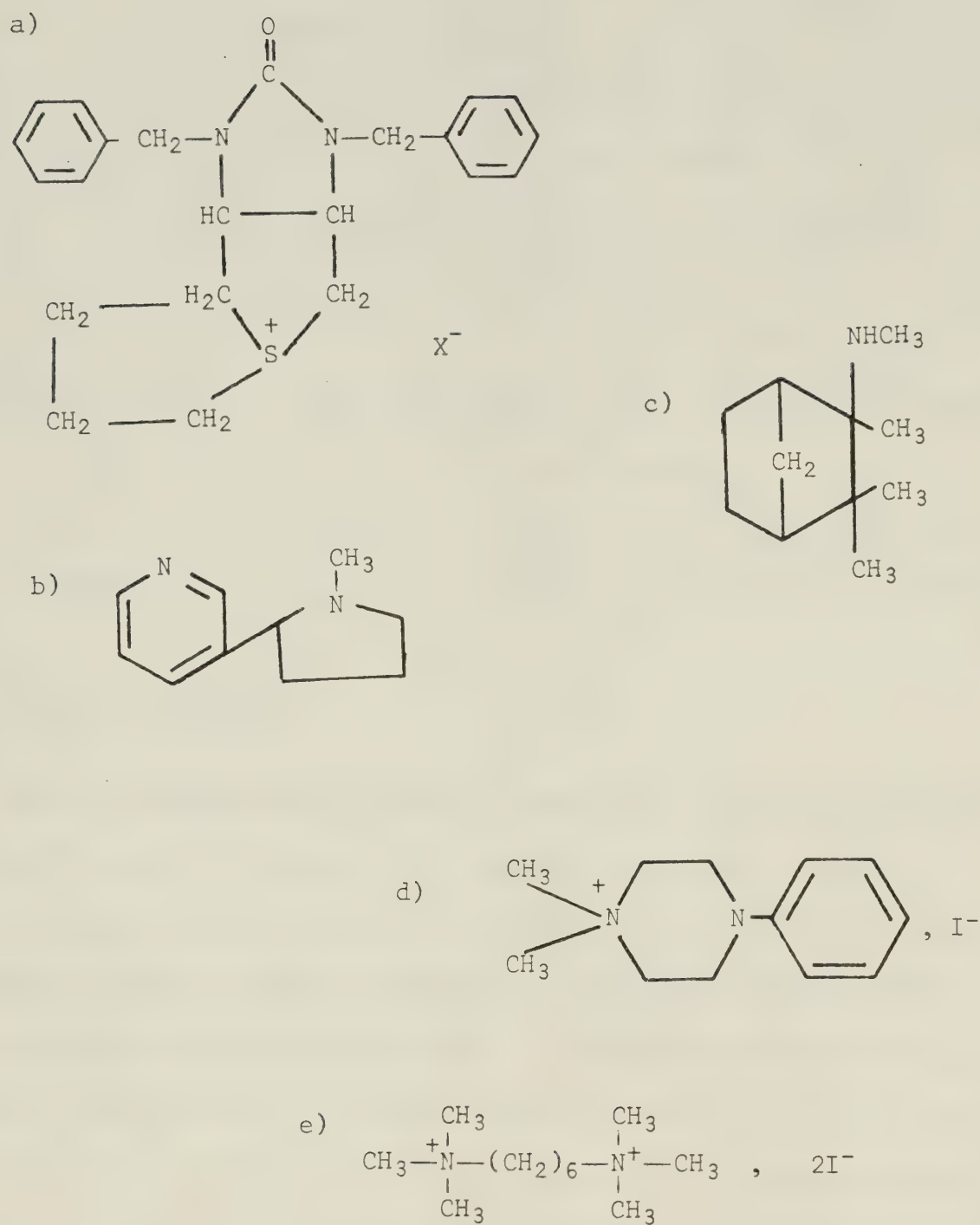


FIGURE 34. Ganglionic blocking agents.

- a) Trimethaphan
 b) Nicotine
 c) Mecamylamine
 d) DMPP
 e) Hexamethonium

X^- = camphorsulfonate

incubated for 10 min in Krebs solution containing phenoxybenzamine (POB), 1×10^{-4} M. In this series of experiments, the length of time that the POB had been dissolved before the incubation was not controlled. After the 10 minute incubation, the tissues were equilibrated for 20 min or until a single dose of agonist again yielded uniform responses. A second dose-response curve for each agonist was then obtained.

Responses to 0.2 or 0.4 ml of 10% KCl in Krebs solution were also determined before and after incubation with POB in most of the tissues. At least four tissues were tested for each treatment.

The methods used to determine the K_D 's from this data were also used in Series B and will be discussed under the heading 'Calculations'.

B. SERIES B EXPERIMENTS

These experiments were primarily undertaken to try and reduce the variation observed in Series A (see Results, chapter 3). Acetyl- β -methylcholine was used in place of acetylcholine as it is only slowly hydrolyzed by AChE. Paired preparations were used in order to reduce the influence of interanimal variation. To try and ensure a relatively constant concentration of the active aziridinium ion, the time between the dissolution of the POB and the incubation of the tissues was also controlled ($32.6 \text{ min} \pm 1.2 \text{ S.E.}, n=41$). Also, the sensitivity (Furchgott, 1966) for responses which were 0.20 of the maximum response were determined before and after POB-blockade in both the test and the control tissues. The experiments were performed in the same manner as Series A. The ganglionic blockers used in this series are denoted by an asterisk in Table 11.

C. CALCULATIONS

Data obtained from the unpaired preparations was used to determine the dissociation constant for the agonist being tested and the fraction of receptors blocked with POB according to the method described by Parker & Waud (1971). The calculations required for this method were done using a Digital Equipment Corporation Mini-computer, Model PDP-11. The program used for these calculations is included in Appendix 5. This method was also used to analyze the dose-response curves obtained with the paired preparations. Initially, each pair of dose-response curves, obtained before and after POB-blockade, was analyzed individually.

However, because of the large variation observed with some of the data (see Results, chapter 3), the method of Parker & Waud (1971) was subsequently applied to:

1. Pairs of dose-response curves obtained by averaging several sets of data, and
2. Responses from several sets of data used simultaneously.

The data was also analyzed using the method described by Furchgott (1966).

The shift of the dose-response curves was measured when $R/R_{\max}$ was $R_{\max}/2$.

Tissue sensitivity was measured before and after POB-blockade when the responses were 0.2 of the maximal response. The sensitivity of the tissue for a given agonist was defined as the reciprocal of the concentration of agonist required to produce the response (Furchgott, 1966).

Changes were judged to be statistically significant on the basis of the standard error of the mean difference (Snedecor, 1967).

RESULTS

The results of these investigations will be presented under four headings:

1. shift of the dose-response curve;
2. fraction of receptors blocked by POB;
3. values of the dissociation constants:
 - a. unpaired tissues
 - b. paired tissues; and
4. tissue sensitivity.

A. SHIFT OF THE DOSE-RESPONSE CURVE

Incubation with POB did not significantly alter the response to KCl, but did produce a shift to the right of the dose-response curves of acetylcholine, carbachol, and acetyl- β -methylcholine. The magnitude of the shifts of the dose-response curves observed in the unpaired tissues are summarized in Table 12. The magnitude of the shift was significantly altered when the tissues were treated with a ganglionic blocking agent, particularly when the agonist was carbachol. However, the variation in the data was very high and it seemed possible that the differences were due to interanimal variation or some other factor rather than the difference between the drugs used. Shifts of the dose-response curves of carbachol and acetyl- β -methylcholine, obtained with paired tissues, are summarized in Table 13. Although the variation remained large, no significant differences were found, suggesting that interanimal variation produced the differences observed with the unpaired data.

TABLE 12

Shifts¹ of Dose-Response Curves in Unpaired Tissues

Treatment	Agonist	
	Acetylcholine	Carbachol
Control	1.86 ± 0.11 (5)	1.99 ± 0.08 (4)
C ₆ 1 × 10 ⁻⁴ M	1.27**± 0.03 (4)	1.36** ± 0.02 (4)
C ₆ 2 × 10 ⁻⁴ M	1.98 ± 0.08 (6)	1.49** ± 0.11 (8)
C ₆ 4 × 10 ⁻⁴ M	1.45 ± 0.09 (4)	1.49** ± 0.09 (3)
Nicotine, 3.2×10 ⁻⁴ M	2.06*± 0.09 (3)	1.51** ± 0.03 (4)
Trimethaphan 3.4×10 ⁻⁵ M	1.34*± 0.18 (3)	1.84 ± 0.02 (4)
Trimethaphan 1.7×10 ⁻⁵ M	1.63 ± 0.11 (6)	1.33** ± 0.07 (4)
Trimethaphan 8.4×10 ⁻⁶ M	1.26*± 0.21 (3)	1.26** ± 0.03 (4)
Mecamylamine 9.8×10 ⁻⁵ M	1.58*± 0.04 (4)	1.56** ± 0.08 (4)

1. Log units ± S.E. (n)

* Significantly different from control, P < 0.05

** Significantly different from control, P < 0.05

TABLE 13

Shifts¹ of Dose-Response Curves in Paired Tissues²

Treatment	Agonist			
	Carbachol		Acetyl- β -methylcholine	
	Control	Test	Control	Test
C ₆ 1 x 10 ⁻⁴ M	1.54 $\pm$ 0.05	1.54 $\pm$ 0.08	1.88 $\pm$ 0.09	1.7 $\pm$ 0.15
C ₆ 5 x 10 ⁻⁵ M	1.43 $\pm$ 0.11	1.53 $\pm$ 0.09	1.54 $\pm$ 0.09	1.55 $\pm$ 0.11
C ₆ 4 x 10 ⁻⁴ M	1.86 $\pm$ 0.18	1.75 $\pm$ 0.31	2.4 $\pm$ 0.15	2.17 $\pm$ 0.12
C ₆ 2 x 10 ⁻⁴ M	1.97 $\pm$ 0.26	1.7 $\pm$ 0.14	2.4 $\pm$ 0.26	1.89 $\pm$ 0.08
*Trimeth. 3.4x10 ⁻⁵ M	1.82 $\pm$ 0.1	1.76 $\pm$ 0.05	2.15 $\pm$ 0.3	2.19 $\pm$ 0.1
*Trimeth. 1.7x10 ⁻⁵ M	1.6 $\pm$ 0.1	1.7 $\pm$ 0.06	2.21 $\pm$ 0.14	2.13 $\pm$ 0.26
DMPP** 6.3x10 ⁻⁵ M		0.46 $\pm$ 0.05		0.51 $\pm$ 0.06

* Trimeth. = trimethaphan

** Five unpaired preparations tested, see text.

1. Log units $\pm$ S.E.

2. n = 4

Control preparations were not used for DMPP as tissues treated with this drug showed increases in $R_{\max}$ after the POB-blockade, and therefore could not be analyzed by the methods used in these investigations (see Figure 35).

B. FRACTION OF RECEPTORS BLOCKED BY POB

The percentages of receptors blocked by POB in the unpaired tissues were determined by the method of Parker & Waud (1971) and are summarized in Table 14. The results were similar to those obtained when the shifts of the dose-response curves were measured. All of the agents tested significantly altered the fraction of receptors occluded by POB when carbachol was the agonist. Only three treatments (C_6 1×10^{-4} M, trimethaphan 1.7×10^{-5} M, and mecamylamine 9.8×10^{-5} M) significantly altered the value obtained when acetylcholine was the agonist.

The results obtained when the fractions of blocked receptors in paired tissues were determined are also similar to the results obtained when the shifts of dose-response curves were measured. These values were calculated using the method described by Furchgott (1966) and also by the method described by Parker & Waud (1971), and are summarized in Table 15. Both methods of calculation produced similar results and only one statistically significant difference was detected. When C_6 5×10^{-5} M was present in the bath, an increase in receptor blockade ($p > 0.05$) was detected by the method of Furchgott (1966) when acetyl- β -methylcholine was the agonist. However, this difference was not confirmed when the data was analyzed by the method described by Parker & Waud (1971).

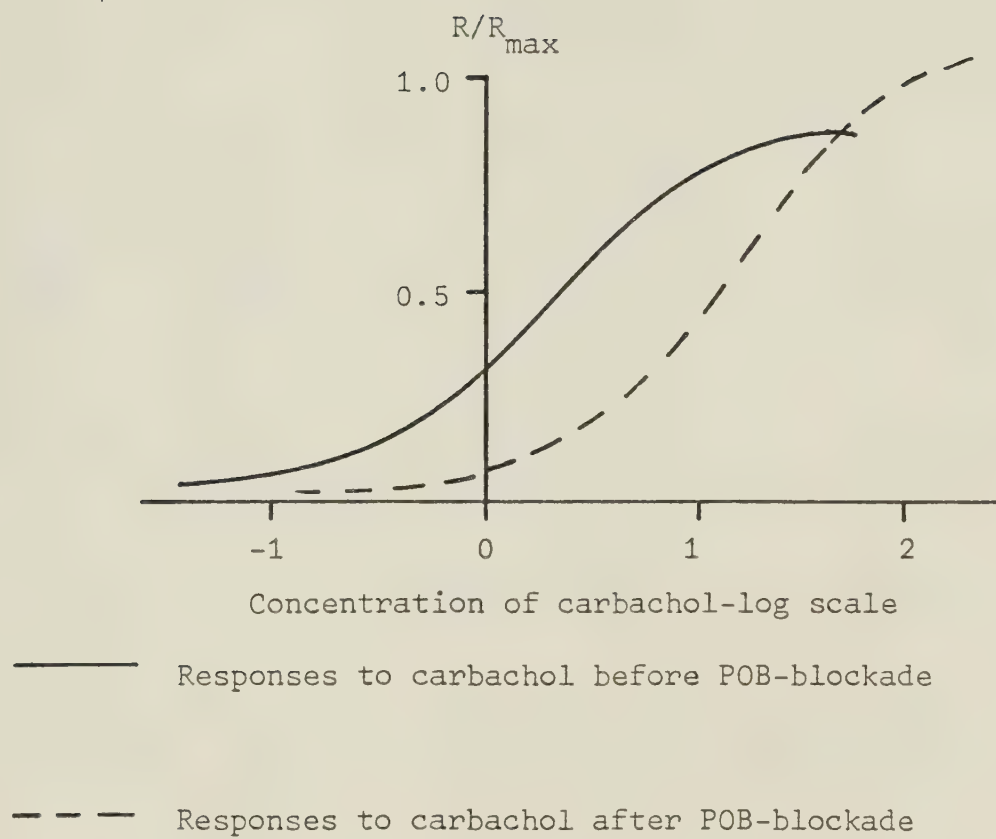


FIGURE 35. Response to carbachol before and after POB-blockade in the presence of DMPP 6.3×10^{-5} M.

TABLE 14

Percent¹ of Receptors Blocked in Unpaired Tissues

Treatment	Agonist	
	Carbachol	Acetylcholine
Control	97.42 ± 0.44 (8)	99.14 ± 0.27 (7)
C ₆ 1 × 10 ⁻⁴ M	92.06** ± 0.39 (3)	93.27* ± 2.15 (4)
C ₆ 2 × 10 ⁻⁴ M	93.38* ± 1.44 (9)	98.5 ± 0.52 (4)
C ₆ 4 × 10 ⁻⁴ M		96.59 ± 2.05 (3)
Nicotine 3.2 × 10 ⁻⁴ M	95.4** ± 0.3 (6)	98.94 ± 0.1 (8)
Trimethaphan 3.4 × 10 ⁻⁵ M	98.19 ± 0.21 (2)	98.68 ± 0.07 (2)
Trimethaphan 1.7 × 10 ⁻⁵ M	90.35** ± 0.68 (3)	97.34** ± 0.3 (6)
Trimethaphan 8.4 × 10 ⁻⁶ M	91.46* ± 2.14 (3)	
Mecamylamine 9.8 × 10 ⁻⁵ M	95.22* ± 0.69 (3)	96.71** ± 0.38 (6)
Mecamylamine 4.9 × 10 ⁻⁵ M	89.74 ± 3.05 (3)	95.4 ± 1.7 (3)
Mecamylamine 2.5 × 10 ⁻⁵ M	91.3* ± 2.22 (5)	98.15 ± 0.56 (3)

1. Mean ± S.E. if > 2 values, otherwise mean ± S.D., calculated according to Furchgott, 1966; n is given in parentheses.

* Statistically different from control, 0.05 < P < 0.01.

** Statistically different from control, P < 0.01.

TABLE 15

Percent¹ of Receptors Blocked in Paired Tissues

Treatment	Carbachol			
	% Block ²		% Block ³	
	Control	Test	Control	Test
C ₆ 2 x 10 ⁻⁴ M	96.42 ± 1.47 (4)	97.12 ± 1.1	95.97 ± 1.78 (3)	97.94 ± 0.95
C ₆ 4 x 10 ⁻⁴ M	92.49 ± 1.31 (4)	95.03 ± 1.4	91.31 ± 2.21 (3)	94.88 ± 1.14
C ₆ 1 x 10 ⁻⁴ M	94.51 ± 2.11 (2)	96.77 ± 0.04	94.54 ± 0.58 (3)	96.72 ± 1.45
C ₆ 5 x 10 ⁻⁵ M	92.00 ± 2.23 (3)	95.72 ± 0.15		
Trimethaphan 3.4x10 ⁻⁵ M	96.68 ± 0.5 (4)	95.96 ± 0.67	96.51 ± 0.62 (4)	97.44 ± 0.58
Trimethaphan 1.7x10 ⁻⁵ M	95.33 ± 1.26 (3)	94.79 ± 0.59	93.65 ± 2.44 (4)	96.7 ± 0.61

Table 15 continued..

TABLE 15 (continued)

Percent¹ of Receptors Blocked in Paired Tissues

Treatment	Acetyl-β-methylcholine			
	% Block ²		% Block ²	
	Control	Test	Control	Test
C ₆ 2 x 10 ⁻⁴ M	98.53 ± 0.2 (4)	97.32 ± 0.54	98.97 ± 0.17 (4)	98.53 ± 0.29
C ₆ 4 x 10 ⁻⁴ M	98.87 ± 0.14 (3)	98.22 ± 0.4	99.47 ± 0.11 (3)	99.09 ± 0.06
C ₆ 1 x 10 ⁻⁴ M	97.63 ± 1.39 (2)	96.2 ± 2.51	98.09 ± 0.29 (3)	94.74 ± 1.75
C ₆ 5 x 10 ⁻⁵ M	88.72 ± 1.32 (3)	93.88 ± 5.7	95.51 ± 3.51 (2)	97.14 ± 0.62
Trimethaphan 3.4x10 ⁻⁵ M	99.26 ± 0.06 (2)	97.37 ± 2.01	98.64 ± 0.97 (3)	98.97 ± 0.44
Trimethaphan 1.7x10 ⁻⁵ M				

1. Mean values ± S.E. if > 2 values, otherwise mean ± S.D.; n given in parentheses. Values could not always be calculated by each method, therefore the value of n is variable.
2. Calculated as described by Furchgott (1966).
3. Calculated as described by Parker & Waud (1971).

C. DISSOCIATION CONSTANTS - UNPAIRED TISSUES

The dissociation constant (K_D) for acetylcholine (ACh) was determined using the method described by Parker & Waud (1971) to analyze data obtained from unpaired tissues. The method was applied to individual pairs of dose-response curves, from before and after POB-blockade, and also to a pair of dose-response curves which represented the average values for several pairs of curves. The values determined by these methods were in reasonable agreement with each other and are summarized in Table 16. The value of K_D was significantly different from the control value in the presence of trimethaphan 1.7×10^{-5} M, mecamlamine (MCA) 4.9×10^{-5} M and MCA 2.5×10^{-5} M, when individual pairs were analyzed. Only the difference with MCA 4.9×10^{-5} M was detected when average values were examined.

In general, the values calculated for the K_D of ACh are larger than those reported in the literature. Furchgott (1966) reported a value of "about 1/8 the K_D of carbachol" (which he reported as $14 \pm 1.7 \mu\text{M}$ S.E. in rabbit aortic strips). Sastry & Cheng (1972) reported a value of $1.08 \mu\text{M} \pm 0.21$ S.E. in the guinea pig ileum. However, in these experiments no effort was made to inhibit the activity of acetylcholinesterase (AChE), and the values of K_D for ACh reported here could therefore have been affected by the activity of the enzyme.

The values for the K_D of carbachol in unpaired tissues were determined with the same methods used for the ACh data and are summarized in Table 17. These values are in broad agreement with the literature (see Table 18). Only tissues treated with C_6 2×10^{-4} M showed a statistically significant difference from control when individual pairs

TABLE 16

Dissociation Constant (K_D) for Acetylcholine

Treatment ³	K_D^1 (μM) calculated by:	
	Method described by Parker & Waud (1971)	Method described by Parker & Waud (1971) applied to averaged dose-response curves ²
Control	100.68 $\pm$ 14.67 (7)	52.81 (8)
C ₆ 1 x 10 ⁻⁴ M	130.37 $\pm$ 47.81 (4)	199.29 (4)
C ₆ 2 x 10 ⁻⁴ M	63.28 $\pm$ 26.93 (4)	
C ₆ 4 x 10 ⁻⁴ M	405.81 $\pm$ 137.69 (3)	
Nicotine 3.2 x 10 ⁻⁴ M	109.64 $\pm$ 27. (6)	178.54 (4)
Trimethaphan 3.4 x 10 ⁻⁵ M	139.65 $\pm$ 11.5 (2)	24.76 (4)
Trimethaphan 1.7 x 10 ⁻⁵ M	37.05* $\pm$ 8.24 (6)	62.19 (8)
Mecamylamine 9.8 x 10 ⁻⁵ M	55.99 $\pm$ 18.34 (6)	41.62 (4)
Mecamylamine 4.9 x 10 ⁻⁵ M	182.72* $\pm$ 12.03 (3)	299.73 (4)
Mecamylamine 2.5 x 10 ⁻⁵ M	42.37** $\pm$ 11.12 (3)	

1. Mean value $\pm$ S.E. if > 2 values, otherwise $\pm$ S.D., n is given in parentheses.
 2. See Dissociation Constants, p. 139.
 3. Unpaired tissues used.
- * Statistically different from control, $P < 0.01$.
- ** Statistically different from control, $0.02 < P < 0.01$.

TABLE 17

Dissociation Constant (K_D) for Carbachol

Treatment ³	K_D^1 (μM) calculated by:	
	Method described by Parker & Waud (1971)	Method described by Parker & Waud (1971) applied to averaged dose-response curves ²
Control	23.80 $\pm$ 4.70 (6)	23.17 (8)
C ₆ 1 x 10 ⁻⁴ M	44.91 $\pm$ 18.21 (3)	55.95 (4)
C ₆ 2 x 10 ⁻⁴ M	74.8* $\pm$ 22.09 (7)	
Nicotine 3.2 x 10 ⁻⁴ M	48.86 $\pm$ 14.91 (4)	
Trimethaphan 3.4 x 10 ⁻⁵ M	47.59 $\pm$ 6.11 (3)	106.65 (4)
Trimethaphan 1.7 x 10 ⁻⁵ M	13.57 $\pm$ 5.51 (3)	67.16 (8)
Trimethaphan 8.4 x 10 ⁻⁶ M	23.42 $\pm$ 4.12 (3)	34.05 (4)
Mecamylamine 9.8 x 10 ⁻⁵ M	45.05 $\pm$ 24.89 (3)	137.78 (4)
Mecamylamine 4.9 x 10 ⁻⁵ M	13.36 $\pm$ 6.83 (3)	16.97 (4)
Mecamylamine 2.5 x 10 ⁻⁵ M	14.15 $\pm$ 3.61 (4)	12.26 (4)

1. Mean value $\pm$ S.E. with n given in parentheses.
 2. See Dissociation Constants, p. 139.
 3. Unpaired tissues used.
- * Statistically different from control, $P < 0.05$.

TABLE 18

Dissociation Constants¹ Reported for Carbachol
at the Muscarinic Receptor

Author(s)	Tissue	K _D ² (μM)
Furchgott, R.F., 1966	rabbit stomach	13.5 ± 3.5
Furchgott, R.F. & Bursztyn, P., 1967	rabbit stomach	15.9 ± 2.4
Lefever, G.S. & Green, R.D., 1975	rabbit stomach	11.7 (range 8.7-15.8)
Fay, F.S. & Singer, J.J., 1977	isolated cells from stomach muscularis of Bufo marinus	9.5 ± 3.7

1. Values determined in absence of C₆.

2. Mean values ± S.E.

of dose-response curves were analyzed. Values based on the averaged curves suggested that several other treatments also caused changes in K_D , e.g. trimethaphan 3.4×10^{-5} M and MCA 9.8×10^{-5} M. However, variation in the data was very high and the changes were not statistically significant.

The major sources of variation in the results for ACh and carbachol were assumed to be interanimal variation and AChE activity. For these reasons, subsequent experiments were performed with paired preparations. Also, acetyl- β -methylcholine was tested instead of ACh as it is only slowly hydrolyzed by AChE. Another likely source of the variation was the possibility of unequal concentrations of the active form of POB. Therefore, in the experiments with paired tissues, the time that the POB was dissolved and allowed to cyclize to the active aziridinium ion was also kept constant. The results obtained when these factors (interanimal variation, AChE activity, and active POB-ion concentrations) were controlled are presented in the next section.

D. DISSOCIATION CONSTANTS - PAIRED TISSUES

The values for the K_D of acetyl- β -methylcholine were determined by the method described by Furchgott (1966) and also by the two methods used to analyze the data from unpaired tissues. The results of these calculations are summarized in Table 19. Variation in the data remained very high, and differences between control and test values were not statistically significant.

TABLE 19
A Comparison of Dissociation Constants (K_D)* Obtained for Acetyl- β -Methylcholine

Treatment ²	Method 1		Method 2		Method 3	
	Control	Test	Control	Test	Control	Test
C ₆ 5 x 10 ⁻⁵ M	8.11 ± 2.89 (3)	8.03 ± 0.42	77.26 ± 14.5 (2)	43.45 ± 1.62		
C ₆ 1 x 10 ⁻⁴ M			96.28 ± 21.59 (3)	306.98 ± 107.62	73.95 (4)	104.96
C ₆ 2 x 10 ⁻⁴ M	50.86 ± 26.26 (4)	102.56 ± 58.	64.78 ± 16.67 (4)	122.56 ± 40.44	51.99 (4)	96.82
C ₆ 4 x 10 ⁻⁴ M	84.18 ± 62.09 (3)	68.57 ± 41.43	131.85 ± 21.19 (3)	161.15 ± 95.25	87.88 (4)	111.78
Trimethaphan 3.4 x 10 ⁻⁵ M			166.46 ± 44.52 (3)	46.30 ± 15.91	135.9 (4)	34.97

1. Mean value ± S.E. if > 2 values, otherwise mean ± S.D., n is given in parentheses.
2. Paired tissues used; K_D could not always be calculated by each method, therefore n is variable.
- Method 1 - method described by Furchgott (1966)
- 2 - method described by Parker & Waud (1971)
- 3 - method 2 applied to data representing the average of several pairs of dose-response curves

* No statistically significant differences found between test and control values for any treatment; statistics applied to methods 1-2 only.

The value of K_D for carbachol was calculated using the same methods that were used to determine the K_D for acetyl- β -methylcholine. In addition, data from several pairs of dose-response curves were analyzed simultaneously using the method of Parker & Waud (1971). The results obtained with each of these methods are summarized in Table 20.

Each of the methods used to calculate the K_D of carbachol produced similar results and the values obtained in control tissues were in close agreement with those reported in the literature (see Table 18). Statistically significant differences from control values were detected when C_6 2×10^{-4} M, C_6 4×10^{-4} M, or trimethaphan 3.4×10^{-5} M were present in the bath and the values of K_D were calculated by the method described by Furchgott (1966). When the values of K_D were determined using the method described by Parker & Waud (1971), only the differences noted when C_6 was present in the bath were statistically significant.

When C_6 was present in the bath, the value of K_D was increased 2-4 times, depending on the method of calculation. The increase did not appear to be dose-related over the range of concentrations examined. Apparent changes in the sensitivity of the tissues to carbachol could account for these results and were therefore investigated.

E. TISSUE SENSITIVITY

The sensitivity of the tissues was measured as the reciprocal of the concentration (in μ M) of carbachol required to produce a response that was 0.02 of the maximal response (Furchgott, 1966). The measurements obtained in the control and test tissues are summarized in Table 21. No statistically significant differences were detected before or after blockade with POB.

TABLE 20

A Comparison of Dissociation Constants (K_D) Obtained for Carbachol

K_D^1 (μM) calculated by:

Treatment ²	Method 1		Method 2	
	Control	Test	Control	Test
$C_6 \ 5 \times 10^{-5} \ M$	$7.37 \pm 0.63 \ (3)$	29.55 ± 9.66		
$C_6 \ 1 \times 10^{-4} \ M$	$9.32 \pm 2.6 \ (2)$	18.25 ± 2.4	$17.42 \pm 4.13 \ (3)$	37.95 ± 13.48
$C_6 \ 2 \times 10^{-4} \ M$	$7.93 \pm 2.6^* \ (4)$	26.91 ± 6.15	$16.85^* \pm 2.22 \ (3)$	76.16 ± 16.33
$C_6 \ 4 \times 10^{-4} \ M$	$9.3 \pm 1.1^* \ (3)$	20.84 ± 2.32	$7.51^* \pm 0.53 \ (3)$	28.1 ± 5.1
Trimethaphan $3.4 \times 10^{-5} \ M$	$12.52 \pm 1.31^* \ (4)$	7.01 ± 0.57	$12.41 \pm 4.2 \ (4)$	13.17 ± 2.27
Trimethaphan $1.7 \times 10^{-5} \ M$	$20.46 \pm 7.83 \ (3)$	18.75 ± 8.47	$16.61 \pm 11.64 \ (3)$	23.61 ± 5.77

Table 20 continued ...

TABLE 20 (continued)
A Comparison of Dissociation Constants (K_D) Obtained for Carbachol

Treatment ²	Method 3		Method 4	
	Control	Test	Control	Test
C ₆ 5 x 10 ⁻⁵ M	7.8 (4)	42.4		
C ₆ 1 x 10 ⁻⁴ M	16.04 (4)	73.85	12.44 (4)	92.79
C ₆ 2 x 10 ⁻⁴ M	7.09 (4)	33.66		
C ₆ 4 x 10 ⁻⁴ M	9.01 (4)	30.79	9.5 (4)	62.75
Trimethaphan 3.4 x 10 ⁻⁵ M	7.87 (4)	16.49	23.94 (4)	19.08
Trimethaphan 1.7 x 10 ⁻⁵ M	9.38 (4)	73.25		

1. Mean value $\pm$ S.E. if > 2 values, otherwise mean $\pm$ S.D., n is given in parentheses.
2. Paired tissues used; K_D could not always be calculated by each method, therefore n is variable.
- Method 1 - method described by Furchgott (1966)
- 2 - method described by Parker & Waud (1971)
- 3 - method 2 applied to data representing the average of several pairs of dose-response curves
- 4 - method 2 applied to data from several pairs of dose-response curves analyzed simultaneously
- * Statistically significant difference between treated and control tissues, $P < 0.05$; statistics only applied to methods 1 and 2.

TABLE 21

Sensitivity¹ (1/ μ M) of Tissues to Carbachol

Treatment ²	Before POB-blockade		After POB-blockade	
	Control	Test	Control	Test
C ₆ 5x10 ⁻⁵ M	7.5 $\pm$ 1.1	14.48 $\pm$ 5.57	0.48 $\pm$ 0.08	0.63 $\pm$ 0.19
C ₆ 1x10 ⁻⁴ M	15.03 $\pm$ 2.36	11.48 $\pm$ 2.56	0.44 $\pm$ 0.5	0.42 $\pm$ 0.06
C ₆ 2x10 ⁻⁴ M	12.87 $\pm$ 2.19	13.47 $\pm$ 2.26	0.34 $\pm$ 0.06	0.39 $\pm$ 0.03
C ₆ 4x10 ⁻⁴ M	5.11 $\pm$ 1.10	5.05 $\pm$ 1.13	0.29 $\pm$ 0.06	0.19 $\pm$ 0.04

1) Mean of four values $\pm$ S.E.

2. No treatment produced statistically significant difference between control and test tissues.

DISCUSSION

A wide degree of variation was observed in the measurements and calculations done during these investigations. Interanimal variation appeared to be an important factor as differences in the magnitude of the shift of the dose-response curves and the values obtained for the percentage of receptors blocked by POB were only statistically significant in unpaired tissues. However, large variations also occurred in some of the data from experiments in which paired preparations were used and other likely sources of variation, e.g. AChE activity and the concentration of POB-ion, had been controlled. Furchgott (1966) reported that the variation in experiments using 'irreversible' blocking agents was much greater than those employing competitive reversible agents, and others have also commented on the wide variation associated with this type of agent (Furchgott, 1954; Bickerton, 1963; Abramson, Barlow, Mustafa & Stephenson, 1969; Abramson, Barlow, Franks & Pearson, 1974). Nevertheless, Parker (1972) reviewed various pharmacological methods of studying binding of drugs to receptors and suggested that true estimates of a molecular reaction were probably being made if independent methods produced the same results. Thus, the consistency among the various methods of calculation used in these investigations suggests that real changes were detected despite the large variation in data. Also, the lack of effect on the response to KCl supports the idea that change occurred at the receptor level rather than through a non-specific depression of muscle contractility.

The magnitude of the shift in the dose-response curve to an agonist is dependent on the fraction of occluded receptors (Furchgott, 1966; Parker & Waud, 1971). No treatment used in these experiments, except

DMPP, produced changes in either parameter. The agreement between the results of these measurements would support the concept of 'spare receptors' for muscarinic agents in the guinea pig ileum. Alternatively, these results can also be interpreted in terms of an allosteric model. In these terms the data suggests that all of the ganglionic blocking agents tested, with the possible exception of DMPP, bind to a site different from that of POB. The shift of the dose-response curve produced by POB was markedly reduced in the presence of DMPP and thus suggests that DMPP and POB may compete for the same site. The actions of DMPP will be discussed in more detail later.

All four methods used to calculate the values of K_D for carbachol detected similar increases in K_D when C_6 was added to the bathing solution. These results are surprising in view of the comments made by Barlow (1975) who suggested that results obtained with the method described by Parker & Waud (1971) were less accurate than calculations done on the assumption that response is linearly related to log dose. However, Barlow (1975) tested only four concentrations of drug (concentrations to produce: $< 25\%$, approximately 25% and 75% , and $> 75\% R_{max}$) and this data would not have been sufficient to accurately define the dose-response curve by the method of Parker & Waud (1971). Several factors could have produced the observed increase in the K_D of carbachol, such as:

1. interanimal variation,
2. a change in the levels of endogenous spasmogens or spasmolytics, or
3. a change in the affinity of the receptor to carbachol.

It is unlikely that interanimal variation could account for the increase in K_D as paired preparations were used.

An apparent increase in sensitivity might produce such results. Such a change in sensitivity might be caused by the inhibition of the effects of endogenous catecholamine release which normally has a relaxant effect on the ileum. This inhibition could arise from nicotinic blockade by C_6 or by POB-blockade of adrenergic receptors. However, in these investigations, no differences in sensitivity to carbachol between test and control tissues were noted before or after blockade with POB. Therefore, it also seems unlikely that sensitivity changes could account for the observed increase in K_D .

In these experiments, the binding of carbachol is changed in the presence of C_6 as evidenced by the increased K_D . It is unclear whether the change is due to a direct effect on the muscarinic receptor or an indirect effect adjacent to the receptor. However, a direct action on the receptor seems unlikely as C_6 did not appear to alter the sensitivity of the tissues to carbachol or to influence the percent of receptors blocked by POB. An indirect effect can be explained in terms of a model in which the receptor can exist in more than one conformation and in which the various conformations have different affinities for ligands (see Figure 32). It seems possible that C_6 may cause more receptors to exist in a high affinity conformation. This could be accomplished by an indirect effect facilitating the conversion of the normal or desensitized conformers to the high affinity state and/or inhibiting the change of receptors in the high affinity conformation to a state of lower affinity.

It is noteworthy that such a model also provides a mechanism for the increases in K_D of some compounds in the presence of C_6 reported, but not explained, by Barlow, Franks & Pearson (1972). Other investigators have reported the ability of C_6 to increase the V_{max} of acetylcholine hydrolysis by AChE, and have suggested that this effect most likely occurs by an allosteric mechanism (Kensler & Elsner, 1951; Belleau, DiTullio & Tsai, 1970; Roufogalis & Wickson, 1975).

In these investigations, the increase in K_D appeared to be selective to the agonist, carbachol. This selectivity would suggest an indirect action of C_6 , as a direct action would presumably affect all agonists binding to the receptor. Geddes, Bogart & Hamilton (1974) also observed that C_6 appeared to selectively alter the effects of some antagonists on the responses to carbachol but not on the responses to acetyl- β -methylcholine. In addition, others have reported that C_6 had no effect on the K_D of acetylcholine in guinea pig ileum or frog rectus preparations (Sastry & Cheng, 1972).

This apparent selectivity may be associated with the preferred conformations of acetylcholine, acetyl- β -methylcholine and carbachol in solution. Acetylcholine and acetyl- β -methylcholine have been shown to have torsion angles ($\tau_{N-C4-C5-O1}$) between 60° and 120° , whereas carbachol is the only known potent muscarinic agonist with a torsion angle ($\tau_{N-C4-C5-O1}$) of approximately 180° (Henderson, 1972).

Although mecamylamine and trimethaphan appeared to have an effect on the K_D for acetylcholine (see Table 16), when calculated as described by Parker & Waud (1971), it seems reasonable to assume that these differences might be due to interanimal variation, as

1. except for mecamlamine, 4.9×10^{-5} M, differences in the K_D were not confirmed by a second method of determining K_D , and
2. effects produced by mecamlamine when carbachol was the agonist tested (see Tables 12 and 14) were eliminated when paired tissues were used (see Tables 13 and 15).

The K_D of agonists in the presence of DMPP could not be determined by the methods used in these investigations, due to the increase in the apparent R_{max} after POB-blockade. Although the inhibition of the POB-induced shift of the dose-response curve can be explained in terms of a simple competition between C_6 and POB for the same binding site, the increase in apparent R_{max} is not readily explained in terms of occupancy or 'spare receptors'. An indirect action, similar to that proposed for C_6 , might provide an explanation. Thus, DMPP may facilitate the formation of more active receptors or drug-receptors complexes.

Thus, one can conclude that hexamethonium produces an effect, at the muscarinic receptor, which results in a change in the affinity of the receptor for carbachol, but not for acetylcholine or acetyl- β -methylcholine. A similar change was not detected with the other ganglionic blocking agents tested, with the possible exception of DMPP. Although affinities between agonists and the muscarinic receptor could not be determined by the techniques used in these experiments when DMPP was present, the data suggests that DMPP may also produce indirect effects at the muscarinic receptor.

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APPENDIX 4

A. DRUGS

Acetylcholine chloride, Fluka

Choline chloride carbamate (carbachol), Fluka

Hexamethonium chloride, Fluka

Acetyl- β -methylcholine chloride, Fluka

Phenoxybenzamine hydrochloride, Smith, Kline and French

Mecamylamine hydrochloride, Merck, Sharp and Dohme

Nicotine dihydrochloride, Baker

1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP), Fluka

Trimethaphan camphorsulfonate injection, Roche

B. CHEMICALS

Calcium chloride, Fisher ACS

Dextrose, Fisher ACS

Magnesium sulfate, Fisher ACS

Potassium chloride, Baker AR

Potassium phosphate monobasic, Fisher ACS

Sodium bicarbonate, Baker AR

Sodium carbonate, Fisher ACS

Sodium chloride, Fisher ACS

APPENDIX 5

Program to Calculate K_D by Method of Parker & Waud (1971)

C: FOCAL-11, LFOCA-A

```

1.01 E
1.02 V E; U E
1.10 A !!! "DRUG-RECEPTOR DISSOCIATION CONSTANT EVALUATION" !!
1.15 A ! "CONTROL CURVE DATA - NUMBER OF POINTS "RN, !
1.20 F I=1, RN; A ! "A ", RA(I), " E ", RY(I)
1.22 A ! "INITIAL ESTIMATE OF K ", RK
1.23 A ! "INITIAL ESTIMATE OF P ", RP
1.25 A ! "TEST CURVE DATA - NUMBER OF POINTS "BN, !
1.30 F I=1, BN; A ! "A' ", RB(I), " E' ", BY(I)
1.35 A ! "REQUIRED PERCENTAGE ITERATIVE PRECISION ", RZ, "%" !!
1.40 D 20; D 5; D 7
1.50 T ! "FINAL K VALUE ", RK
1.60 T ! "FINAL M VALUE ", RM
1.70 T ! "DISSOCIATION CONSTANT USING IRREVERSIBLE "
1.71 T "ANTAGONIST ", RK-RM
1.75 T ! "PERCENT OCCLUSION OF RECEPTORS BY IRREVERSIBLE "
1.76 T "ANTAGONIST "%4.02, 100*(1-RM/RK), " %"
1.80 T ! "DISSOCIATION CONSTANT FOR PARTIAL AGONIST "%, RK
1.90 T ! ! ! ! ; Q

5.10 T ! "PAIRED DOSE VALUES" !! " CONTROL TEST" !
5.15 S R1=0; S R2=0; S R3=0; S R4=0; S R6=0
5.20 F RI=1, BN; D 6
5.25 S BS=(R6*R3-R1*R2)/(R6*R4-R1*2)
5.30 S BI=(R2*R4-R3*R1)/(R6*R4-R1*2)
5.35 S RK=BS/BI
5.40 T ! "INITIAL ESTIMATE OF PARAMETER K ", RK, !

6.10 S R5=BY(RI)/(RM-BY(RI)); S R5=FSBR(15, R5)/RP
6.15 S RA(RI)=RK*FSBR(14, R5)
6.20 T %, RA(RI), " ", RB(RI), !
6.25 S RR=RA(RI)*4
6.30 S R1=R1+RR/RB(RI); S R2=R2+RR/RA(RI)
6.40 S R3=R3+RR/(RB(RI)*RA(RI)); S R4=R4+RR/RB(RI)*2
6.50 S R6=R6+RR

7.01 T ! "HYPERBOLIC PARAMETERS" !!
7.05 F RJ=0, 1; F RW=0, 2; S AA(RJ, RW)=0
7.10 F RI=1, BN; D 8
7.20 S AV=1; D 26; S RM=AX(0); S RL=RK+AX(1)/RM
7.40 I (100*FABS((RL-RK)/RK)-RZ)7.6
7.45 T "% ", RL, " M ", RM, !
7.50 S RK=RL; G 7.05
7.60 S RK=RL; R

8.10 S RX(0)=RB(RI)/(RB(RI)+RK)
8.20 S RX(1)=-RB(RI)/(RB(RI)+RK)*2
8.30 F RJ=0, 1; S AA(RJ, 2)=AA(RJ, 2)+RX(RJ)*RA(RI); D 9
8.40 R

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9.10 F RW=0,1; S AA(RJ,RW)=AA(RJ,RW)+RX(RJ)*RX(RW)
9.20 R

14.10 I (&†2-.01)14.2; S &=&/2; D 14; S &=&†2; R
14.20 S &=1+&+&†2/2+&†3/6+&†4/24+&†5/120+&†6/720

15.10 I (&†2-2.04*&+1)15.2; S &=FSQT(&); D 15; S &=2*&; R
15.20 S &=(&-1)/(&+1); S &=2*(&+&†3/3+&†5/5+&†7/7)

20.01 T !"CONTROL CURVE PARAMETERS"!!
20.02 F RJ=0,2; F RW=0,3; S AA(RJ,RW)=0
20.03 S R5=RP*FSBR(15,RK); S R5=FSBR(14,R5)
20.04 S R6=(RP-1)*FSBR(15,RK); S R6=FSBR(14,R6)
20.06 F RI=1,RN; D 21
20.10 S AV=2; D 26; S RM=AX(0); S RL=RK+AX(1)/RM; S RQ=RP+AX(2)/RM
20.15 T "K "%,RL," P "%,RQ," M "%,RM,!
20.20 I (100*FABS((RQ-RP)/RP)-RZ)20.3,20.35,20.35
20.30 I (100*FABS((RL-RK)/RK)-RZ)20.4,20.35,20.35
20.35 S RP=RQ; S RK=RL; G 20.02
20.40 S RK=RL; S RP=RQ; R

21.01 S R4=RP*FSBR(15,RA(RI)); S R4=FSBR(14,R4)
21.04 S RX(0)=R4/(R4+R5)
21.05 S RX(1)=-RP*R4*R6/(R4+R5)†2
21.06 S RX(2)=R4*R5*FSBR(15,RA(RI)/RK)/(R4+R5)†2
21.10 F RJ=0,2; S AA(RJ,3)=AA(RJ,3)+RX(RJ)*RY(RI); D 22
21.20 R

22.10 F RW=0,2; S AA(RJ,RW)=AA(RJ,RW)+RX(RJ)*RX(RW)

26.10 F AK=0,AV; S AR(AK)=AK+1
26.13 S AI=-1; S AL=AV+1
26.15 S AM=0
26.17 F AJ=0,AV; F AK=0,AV; D 27
26.19 S AR(AP)=0
26.21 F AK=0,AL; S AA(AP,AK)=AA(AP,AK)/AM
26.23 F AJ=0,AV; D 28
26.25 S AI=AI+1; I (AI-AV)26.15,26.27,26.15
26.27 F AJ=0,AV; F AK=0,AV; D 29
26.30 R

27.10 I (AR(AJ))0,27.3,27.15
27.15 I (FABS(AA(AJ,AK))-FABS(AM))27.3
27.20 S AM=AA(AJ,AK); S AP=AJ; S AQ=AK;
27.30 R

28.10 I (AJ-AP)28.2,28.4,28.2
28.20 S AE=AA(AJ,AQ)
28.30 F AK=0,AL; S AA(AJ,AK)=AA(AJ,AK)-AA(AP,AK)*AE
28.40 R

29.10 I (1E-6-FABS(AA(AJ,AK)))29.2; R
29.20 S AX(AK)=AA(AJ,AL); R

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